INTERRELATION BETWEEN α-TOCOPHEROL AND PROTEIN METABOLISM

IV. THE CURE AND PREVENTION OF STOMACH ULCERS IN RATS [1]

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THREE FIGURES

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Hyperplastic ulcer-like lesions in the rumen of the stomach of rats have been noted in a wide assortment of nutritional dysfunctions. The literature on this subject has been reviewed by Zucker, Berg and Zucker ('45), who have shown that the protein level in the diet is of critical importance in this connection. They suggest "the possibility of a specific factor important for the prevention of lesions of the rumen which may be either an amino acid labile to commonly employed methods of purifying casein, or an unknown factor associated with crude casein."

In a previous paper from this laboratory, Jensen ('46) noted that α-tocopherol prevented rumen lesions in rats recovering from vitamin A deficiency. At the time this was interpreted as another demonstration of the well-known sparing action of vitamin E on vitamin A. However, further work revealed that the lesions occurred even with high intakes of vitamin A (Harris, Hove, Mellott and Hickman, '47). Thus, the action of tocopherol could not be explained simply as making more vitamin A available to the animal, although

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local action in the stomach seemed indicated by the failure of injected tocopheryl phosphate to protect. It was also shown that lesions which developed during the slow cure of pyridoxine-deficient rats were prevented by tocopherol. Similar results were found in the case of essential fatty acid deficiency in rats.

The role of tocopherol in preventing stomach ulcers due to various nutritional maladjustments is obscured by lack of knowledge of the etiology of such lesions. Before the mechanism of tocopherol participation can be understood more experimental data need to be obtained. The present investigation, which examines the effect of protein or calorie restrictions, or both, on stomach ulcer development in rats, attempts to supply some of the facts regarding such lesions.

DIETS AND METHODS

Three basic approaches have been adopted in this study of the influence of tocopherol on gastric ulcers: (1) total starvation of adult rats followed by controlled repletion; (2) semi-starvation of adult rats: the influence of protein content and type of fat in the diet; (3) maintenance of adult and weanling rats on low-protein, adequate-calorie diets.

The composition of the diets used is given in table 1; they were vitamin E-free. In calculating the caloric values of the diets it has been assumed that sucrose and crude casein contribute 4.1 calories and fat 9 calories per gram. Thus the diets with 10% fat contain approximately 45 calories per 10 gm. They were made up at frequent intervals and stored in a cold room (0–5°C.) The desired quantities of diet were weighed out daily on a triple-beam balance and offered to the rats in open dishes. After becoming accustomed to a single meal a day each animal ate his allotment within half an hour, with no spillage.

Vitamin E, 1 mg of pure *d*,*α*-tocopherol, was fed daily in two drops (50 mg) of olive oil. In a few cases higher levels

(up to 10 mg) were fed, but since no differences were noted at the various levels these results have been grouped together to condense the data. The tocopherol supplement was given 2 to 4 hours after the daily food allotment.

TABLE 1

Composition of diets

	DIET NO.				
	56	57	58	59	60
			%		
Crude casein	22.5	22.5	22.5	22.5	20.0
Sucrose	63.0	63.0	63.0	63.0	76.0
Salt mixture USP XIII [1]	4.5	4.5	4.5	4.5	4.0
Lard	10.0	0	10.0	0	0
Wesson oil	0	0	0	10.0	0
Hydrogenated coconut oil	0	10.0	0	0	0
			mg/kg of diet		
Thiamine chloride [2]	2	10	10	10	10
Riboflavin [2]	2	10	10	10	10
Pyridoxine [2]	2	10	10	10	10
Ca pantothenate [2]	10	25	25	25	25
Niacin [2]	20	100	100	100	100
i-Inositol [2]	100	500	500	500	500
Choline chloride [2]	500	1,000	1,000	1,000	1,000
2 Me 1,4 naphthoquinone [2]	5	5	5	5	5
Vitamin A [3]	2,000 units		Fed by dropper once		
Vitamin D [4]	500 units		weekly in olive oil		

[1] With $ZnCO_3$, 0.1%; $CuSO_4 \cdot 7\ H_2O$, 0.08%; $MnSO_4$, 0.02%.

[2] Merck synthetic vitamins; these were thoroughly ground with a small quantity of the casein before being incorporated in the diets.

[3] Concentrate of natural esters of vitamin A.

[4] Delsterol.

The rumen lesion

In general, the lesions produced by the various dietary techniques were grossly and histologically similar to those described by Pappenheimer and Larimore ('24).

The stomach of a rat dying of starvation is compared with a normal stomach in figure 1. Photomicrographs of sections made through the typical lesions of the rumen and

fundus are shown in figure 2, A and B.[3] The black spots in the fundus are typical in rats dying of stravation. Fundic lesions of this type have not been observed under any of the other experimental conditions used in this laboratory.

Examination was conducted by removing the stomachs, cutting along the greater curvature, washing, and spreading out. Three aspects were considered in expressing the results: the incidence of lesions (i.e., the percentage of rats in the group showing lesions); the severity of the condition as

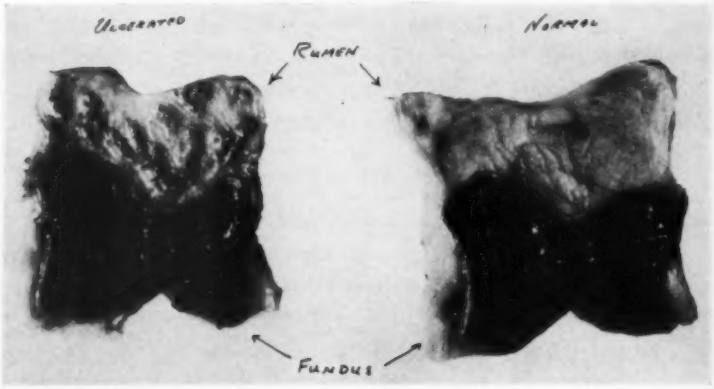

Fig. 1 The stomach of a rat dying of starvation compared with a normal stomach.

judged by the average number of individual craters; and the severity of the condition as judged by the size of the craters. Gross inspection only was used in this work. No satisfactory method for expressing crater size was devised, so the results have been given simply in terms of incidence and average number of craters per rat among those rats showing lesions.

[3] We greatly appreciate the courtesy and cooperation of Dr. K. E. Mason, University of Rochester School of Medicine and Dentistry, who prepared the histological specimens, and of Dr. R. P. Loveland, Eastman Kodak Company, who photographed the tissue sections.

2A

2B

Fig. 2A Photomicrograph of section through a hyperplastic lesion in the rumen of a rat. Note the thickening of the epithelial layer, with complete rupture and sloughing of thin layer in the center. × 50.

Fig. 2B Photomicrograph of section through a starvation-induced fundic lesion. The ghostlike structure at the tip of the fold corresponds with a black spot visible grossly. × 50.

The rate of cure of lesions after total starvation for 8 days

Weanling albino rats were fed to exactly 250 gm body weight on diet 58 (table 1), ad libitum. Food was then withheld and the rats starved for 8 days. The weight losses were 27 to 29% of the initial weights. By examining a few rats daily during this period it was found that lesions in the rumen began to appear on the 5th day and increased rapidly in severity until on the 8th day the rats had an average of 13.8 craters per rumen. The wall of the rumen was greatly thickened with a very marked increase in vascularization and enlargement of blood vessels. Fundic lesions had not yet appeared. Survival time averaged 18 days for a group continued on this regimen.

At the end of the 8th day of starvation half the rats were given an α-tocopherol supplement amounting to 1 mg daily. All were then given 40 Calories (8.9 gm) of diet 58 daily. This controlled rehabilitation was used because preliminary experiments had shown that ad libitum feeding during restoration resulted in complete cures of the stomach ulcers within 4 days.

The rates of cure of the rumen lesions with 40 Calories per day are shown in figure 3. The numerals in parentheses indicate the number of animals autopsied after 7, 14 and 21 days of repletion. The average weight change for the E-free and E-supplemented groups, respectively, during this rehabilitation were: -6.0 and -5.1 gm after 7 days; $+7.3$ and $+10.3$ gm after 14 days; and $+16.1$ and $+23.1$ gm after 21 days. The standard errors for the 21-day weight gains were ± 2.78 and ± 2.04, respectively. Applying the standard Fisher t test for significance, $D/E_D = 2.03$, while the t value for $P = 0.05$ is 2.13. Thus the difference in weight gain between the two groups falls just short of significance at the 5% level.

Tocopherol given during repletion aids materially in increasing the rate of cure of the rumen lesions. Whether this

is a primary reaction or a secondary effect due to better utilization of the available food is not evident from these data.

The prevention of rumen lesions produced during semi-starvation

Male albino rats were placed on diets 56, 57, 58, 59 or 60, ad libitum. The composition of these diets, differing in the type of fat at the 10% level, is given in table 1. Diets 56 and

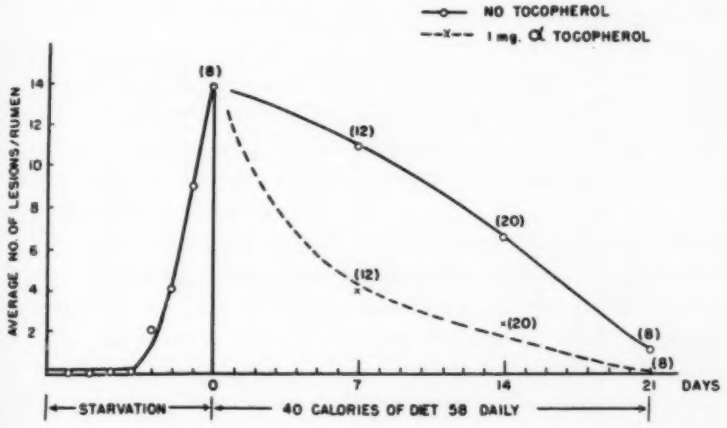

Fig. 3 The effect of tocopherol on the rate of cure of rumen lesions produced by 8 days' starvation of adult male rats. Figures in parentheses indicate number of animals autopsied.

58 contained lard, diet 57 hydrogenated coconut oil, diet 59 cottonseed oil,[4] and diet 60 was fat-free. Diets 56 and 58 differed in the amount of B-complex vitamins they contained. When the rats reached 200 gm in body weight they were transferred to a daily intake of 20 Calories (4.5 gm of the fat-containing diets and 5.0 gm of of the fat-free diet). Some of the animals on each of these diets had been receiving tocopherol during their growing phase but this was discon-

[4] Wesson.

TABLE 2

The effect of tocopherol and type of fat (10%) on rumen lesion production in 200-gm rats subjected to semi-starvation (20 calories daily) for 40 days

DIET NO.	DAILY α-TOCOPHEROL SUPPLEMENT	NO. OF RATS	AVERAGE WEIGHT LOSS IN 40 DAYS	RUMEN LESIONS	
				Incidence	Severity
	1.0 mg		*gm ± S.E.*	%	*craters/rat* [1]
58 (Lard)	None	13	81.4 ± 2.37	39	2.0
	During restriction	21	78.2 ± 2.06	9	1.0
	Prior to restriction	21	83.9 ± 3.03	5	1.0
56 (Lard and low B-complex)	None	10	100.2 ± 1.55	80	5.4
	During restriction	12	98.4 ± 2.52	8	4.0
	Prior to restriction	4	100.8 ± 2.66	100	2.2
59 (Wesson oil)	None	10	90.4 ± 1.62	10	2.0
	During restriction	10	87.4 ± 2.19	10	1.0
	Prior to restriction	10	90.0 ± 1.93	10	1.0
60 (Fat-free)	None	8	81.0 ± 3.76	0	0
	During restriction	8	82.5 ± 3.40	0	0
	Prior to restriction	8	84.7 ± 3.80	11	1.0
57 (Coconut oil, hydrogenated)	None	10	67.9 ± 3.02	60	2.1
	During restriction	9	71.6 ± 2.96	45	1.8
	Prior to restriction	10	77.8 ± 3.87	50	2.4

[1] Expressed as average number of craters per rat of those showing lesions.

tinued during semi-starvation. Others were given a tocopherol supplement only during the semi-starvation period. Still others were kept vitamin E-free throughout the experiment.

Semi-starvation of the adult rats was continued for 40 days after which they were killed and their stomachs examined. The results with respect to weight loss during the 40 days of semi-starvation and the incidence and severity of the lesions are given in table 2.

From these data it is evident that stomach lesions were common on the lard diets but preventable to a large extent by α-tocopherol fed during semi-starvation. The lesions on diet 56 (low B-complex) were much more severe than those on the high B-complex diet, 58. Adequate vitamin E intake during growth prior to semi-starvation gave good protection against ulcer development. This was evident on diet 58 and to a lesser degree on diet 56.

On the cottonseed oil diet (59) no significant lesion development occurred. This was undoubtedly due to the tocopherol furnished by this oil (approximately 0.45 mg/rat/day).

On the fat-free diet no lesions developed. This seemed to indicate that tocopherol-free fats were essential to lesion development. However, on hydrogenated coconut oil, which contains no tocopherol or unsaturated fat, abundant lesions developed. Tocopherol, at least in the quantity used in this experiment, did not prevent these lesions. Other investigators (Miller and co-workers, '44) have reported that hydrogenated coconut oil induced anomalous results, not influenced by α-tocopherol, in their feeding experiments.

The rumen lesions resulting from a restricted intake of diets 58 or 56 may have been induced by the calorie restriction or by the reduced protein intake of these regimens. To check this point the crude casein content of diet 58 was varied from 0 to 80%, with the caloric level maintained by alteration in the sucrose content of the diet. No other change was made. These new diets were designated 581 to 587. The

TABLE 3

The effect of casein, tyrosine and tocopherol on rumen lesion production in 200-gm rats subjected to semi-starvation (20 calories daily) for 40 days

DIET NO.	CRUDE CASEIN IN DIET	DAILY α-TOCOPHEROL SUPPLEMENT	NO. OF RATS	AVERAGE WEIGHT LOSS IN 40 DAYS	RUMEN LESIONS	
					Incidence	Severity
	%	*mg*		*gm*	%	*craters/rat* [1]
581	0	0	5	90.1	100	7.4
		1	5	90.3	60	3.3
582	5	0	5	87.2	100	2.6
		1	5	84.0	60	1.7
583	10	0	5	78.4	40	1.0
		1	5	79.2	20	1.0
584	20	0	5	77.2	0	0
		1	5	79.2	0	0
585	40	0	5	82.8	0	0
		1	5	76.3	0	0
586	80	0	5	89.5	0	0
		1	5	83.0	0	0
587	15	0	10	85.4 ± 2.09 (S.E.)	70	1.4
		1	10	83.7 ± 1.82	10	1.0
587A	15 + 3% L-tyrosine	0	10	81.8 ± 3.02	40	1.5
		1	10	87.1 ± 3.41	20	2.0
587B	15 + 9% L tyrosine	0	9	76.0 ± 1.60	22	1.5
		1	9	67.7 ± 2.90	11	1.0

[1] Expressed as average number of craters per rat of those showing lesions.

procedure in handling the rats was the same as previously indicated except that all the rats were raised to a weight of 200 gm on diet 58 fed ad libitum.

From the data in table 3 it is apparent that increasing the crude casein content of the diet completely protects against rumen lesion development during semi-starvation. Rats on a restricted intake of diet 584 showed no lesions, although this diet was practically equivalent to the diet 58 used in the previous experiment. The reason for this may be that the crude casein used was from a different source. Rats on the diets sufficiently low in casein to permit the development of lesions were protected significantly by the α-tocopherol supplements.

Included in table 3 are some data on the effect of L-tyrosine added to a low-protein diet. On diet 587 (15% crude casein) the lesion incidence was 70%. Addition of 9% L-tyrosine to this diet at the expense of sucrose reduced the incidence to 22%. Three per cent L-tyrosine gave an intermediate value.

Ulcers induced by low-protein, adequate-calorie diets

Weanling rats, fed for 4 weeks a diet containing 5% purified casein[5] but otherwise similar to diet 582, showed extremely severe lesions which were not appreciably prevented by 1.0 mg daily of α-tocopherol. Omission of the choline or inositol or both from the diet, or the inclusion of L-methionine up to a level of 0.15%, had no influence on the lesions, either with or without tocopherol. On the other hand, L-cystine at a level of 0.5% of the diet drastically reduced the severity of the lesions. This is similar to results noted by Sharpless ('36).

It had been observed in a previous paper (Hove, '46) that adult rats maintained on an E-free, 5% casein diet for 20 weeks developed a very high incidence of severe stomach lesions, while similar rats receiving α-tocopherol showed a

[5] Labeo.

low incidence and low degree of severity of lesions. This was confirmed by subsequent work (Hove and Harris, '47), in which it was noted that severe forestomach lesions developed in 4 of 6 rats fed for 12 weeks on a 5% casein, vitamin E-low diet, while of 6 controls receiving tocopherol, only one rat showed lesions. Rats fed a 10% casein, vitamin E-low diet for 12 weeks had no grossly visible ulcers. However, after 6 to 8 months, 10 of 12 survivors showed marked hyperplastic lesions. No ulcers occurred in rats maintained for periods up to one year on vitamin E-low diets with a normal (20%) casein level.

DISCUSSION

In our previous report (Harris, Hove, Mellott and Hickman, '47), ulcer-like lesions of the rumen were found in rats recovering from deficiencies of vitamin A, pyridoxine or essential fatty acids. Our present experiments describe similar lesions developed by starvation, semi-starvation and low-protein diets. Tocopherol was highly beneficial in preventing or curing the lesions, as judged by decreased incidence and severity.

This uniformity of action of tocopherol in such widely differing nutritional dysfunctions seems to indicate a common etiology for the lesions. Several factors indicated that the action of tocopherol was localized in the stomach. Thus, injected tocopheryl phosphate did not protect, and a fat with some unsaturation (lard as compared with hydrogenated coconut oil or no fat) was necessary in the diet to demonstrate the effect of tocopherol. On the other hand, some protection was conferred on animals given sufficient tocopherol early in life to insure adequacy of vitamin E nutrition but deprived of tocopherol during the experimental period of ulcer development.

If the action of tocopherol is localized in the stomach, it presumably acts as an anti-oxidant in some metabolic mecha-

nism. In this respect it is interesting to note that Roffo ('42) observed ulcers and "cancers" in the stomachs of rats fed oxidized oils. He ascribes the effect to production of a carcinogenic oxycholesterol. Hove and Harris ('46) have shown that linolate is destroyed in the gastrointestinal tract, presumably by oxidation with the formation of peroxides, and that tocopherol can prevent this destruction.

The etiology of the rumen lesions may involve a balance between the presence of local irritants and the resistance capacity of the epithelial cells of the rumen to this irritation. Besides the usual irritants such as hydrochloric acid or enzymes, it is possible that fat peroxides have an irritant action.

An increase in irritant concentration or a decrease in the resistance power of the cells, or a combination of the two, may dispose to lesion formation. In deficiencies of vitamin A, pyridoxine, essential fatty acids and protein, it is well known that the integrity of epithelial cells are affected, and these deficiencies undoubtedly reduce resistance to local irritants.

Tocopherol may act by reducing the concentration of total irritants through preventing peroxide formation. The beneficial effect of high-casein diets in preventing lesions may similarly be due to a reduction in total irritants, in this case by neutralizing hydrochloric acid.

This working hypothesis appears to fit most of the facts, but the possibility of the direct physiological involvement of tocopherol and protein is by no means ruled out.

SUMMARY

1. Ulcer-like lesions of the rumen have been developed by starvation, semi-starvation and low-protein diets in adult rats reared on vitamin E-free diets.

2. α-Tocopherol greatly increased the rate of cure of the stomach lesions produced by total starvation for 8 days when repletion was carried out with 40 Calories of an E-free diet daily.

3. α-Tocopherol reduced the incidence and severity of rumen lesions produced by semi-starvation (20 Calories daily for 40 days) on lard diets. Lard in the diet seemed essential for this effect. Hydrogenated coconut oil induced ulcers but these were not affected by 1.0 mg daily of tocopherol. No rumen lesions were produced when fat-free diets were fed. Increasing the crude casein or adding tyrosine reduced or prevented lesion formation during semi-starvation, even in the absence of vitamin E.

4. Weanling rats on a low-casein diet, fed ad libitum for a short period, developed marked lesions which were preventable by cystine by not by the level of tocopherol fed (1.0 mg/day). Adult rats on a low-protein, low-E diet for long periods developed ulcers which were preventable by α-tocopherol.

5. A working hypothesis has been advanced to explain the beneficial action of tocopherol on stomach ulcers induced by dietary variations.

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EFFECT OF THE PREPARTAL DIET OF THE COW ON THE PLACENTAL AND MAMMARY TRANSFER OF TOCOPHEROLS TO THE CALF [1]

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Although the placental and mammary transfer of tocopherols has been studied with sheep, goats and swine (Whiting and Loosli, '48), reports on similar studies with cattle have not been found in the literature. Parrish et al. ('47) have shown that the colostrum of the dairy cow is a rich source of tocopherols, the concentrations of which decrease during the transition to normal milk, and that the tocopherol levels of the early mammary secretions are augmented by increasing the prepartal intake of this vitamin by the dam. Studies of serum tocopherol concentrations in dairy cattle (Latschar et al., '49) revealed characteristic levels, which apparently are associated with stages of terminal gestation and early lactation. It was found further that both levels and trends of this vitamin in the serum were altered by tocopherol supplementation. The present report includes observations on the placental and mammary transfer of tocopherols in dairy cattle subjected to several different prepartal dietary regimens.

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EXPERIMENTAL PROCEDURES

During the terminal 4 to 6 weeks of gestation, dairy cows were fed a typical barn ration, which included alfalfa hay, Atlas sorgo silage and a concentrate mixture. In addition, some of the cows were given supplements of tocopherols and of vitamin A (table 1). Further details of the feeding and

TABLE 1

Concentrations of tocopherols in blood serum collected from dairy cows after parturition and from newborn calves before colostrum ingestion

PREPARTAL SUPPLEMENT OF DAM [1]			NUMBER OF ANIMALS	TOCOPHEROL LEVELS IN BLOOD SERUM		AVERAGE SERUM TOCOPHEROLS
Vitamins	Daily amounts			Dam	Calf	
	28–14 days	13–0 days				
				μg/100 ml	*μg/100 ml*	*ratio dam/calf*
None	..	..	7 [2]	276 ± 27 [3]	44 ± 31	6.3
None	..	..	1 [4]	347	25	13.9
Vitamin A alcohol or ester	500,000 I.U.	1,000,000 I.U.	14	255 ± 86	53 ± 31	4.8
Vitamin A alcohol + α-γ tocopherols [5]	500,000 I.U. + 0.5 gm	1,000,000 I.U. + 1.0 gm	6	350 ± 31	74 ± 36	4.7
α-γ tocopherols	0.5 gm	1.0 gm	1	396	43	9.2
α-γ tocopherols	5 gm	5 gm	1	817	43	19.0
Mixed tocopherols	4 gm	4 gm	1	1003	68	14.7
Mixed tocopherols	10 gm	10 gm	3	1101 ± 235	79 ± 20	13.9

[1] All supplementation discontinued at parturition.

[2] Results from 5 cows and 7 calves.

[3] Standard deviation.

[4] Cow milked throughout gestation.

[5] α-γ tocopherols contained either 50% or 90% of the γ form; the tocopherols were in the free unesterified state.

management of the cows have been reported (Parrish et al., '47; Latschar et al., '49).

Calves from the experimental cows were hand-fed colostrum from their respective dams during the first three postnatal days and whole, mixed herd milk during the next 25 days. After the 14th day, prairie hay and a grain concentrate were fed ad libitum. Colostrum and milk were given morning and evening at the rate of 1 lb. daily per 10 lb. body weight of the calf. Occasionally, after the first week, some excess colostrum was included in the whole milk, but this constituted only a small portion of the total consumption.

Shortly after parturition, samples of venous blood were obtained from the cow and from the calf. The first sample from the latter was collected before colostrum feeding. Additional blood samples were drawn the mornings of the first, second and third days after birth from all calves except those sacrificed for studies of the vitamin A content of the livers. For several calves sampling was continued at weekly intervals through the 28th day. The blood samples were allowed to clot and then centrifuged to obtain the serum, which was stored in tightly closed bottles in the dark at 4°C. whenever analyses could not be made immediately. The method employed for determination of tocopherols (Latschar et al., '49) was essentially the procedure of Quaife and Biehler ('45).

RESULTS

Placental transfer of tocopherols

Calves born of cows having a normal dry period and receiving unsupplemented barn rations had initial serum tocopherol concentrations averaging 44 μg per 100 ml, a level approximately one-sixth that of the dam at the same time (table 1). Straumfjord and Quaife ('46) reported that the ratio of plasma tocopherols in the blood of human mothers to those in the cord blood of the infant at time of delivery was 5.7 : 1, a value only a little lower than that found in the present investigation. Vitamin A supplements added to the

diet of the dam had no apparent effect on tocopherol levels in either cows or calves.

Tocopherol concentrations in the serum of the cows were increased by daily oral administration of various levels of this vitamin during the concluding stages of gestation, the level in cows receiving 10 gm of supplement being about 4 times higher than that in unsupplemented animals (table 1). There was a tendency for supplementation of the dam to increase tocopherol levels in newborn calves, but the higher levels of supplementation were no more effective than the 0.5–1 gm level (table 1). The variations observed within groups of newborn calves renders these increases in tocopherol concentration of questionable significance. The ratio of tocopherol levels in the serum of cows to those in calves was widened markedly when the dams received the larger quantities of tocopherols. An exception is noted in the case of the non-supplemented cow milked throughout gestation, but further studies are required before the significance of this can be established.

As far as could be determined, the amount of tocopherols in the blood serum of calves was not related to congenital vitality.

Mammary transfer of tocopherols

Changes in the serum tocopherols of calves during the first month of life are shown in table 2. In all cases, concentrations of this vitamin increased following the ingestion of colostrum. The tocopherol levels in calves from dams that received either barn rations or such rations supplemented with vitamin A only during the terminal stages of gestation increased more than 4-fold by the third day, most of the change occurring during the first two days. The concentrations in the calf of the cow (H-126) that was milked throughout gestation did not increase to so high a level after consumption by the calf of mammary products from its dam as did those of other calves ingesting colostrum. Serum tocopherol levels in calves from dams that received

vitamin A and 0.5–1 gm of tocopherol supplements prepartally were increased approximately three-fold by the second day after birth; whereas those in calves of cows receiving 4 to 10 gm of supplement during the last month of gestation were increased 6- to 9-fold by the second day. In contrast with the upward trend in calves from unsupplemented dams, the levels of tocopherols in calves from tocopherol-supplemented cows declined after the second day.

In several instances tocopherol levels in the blood serum from calves on the second, third or 4th day were as high as, or higher than, those in samples collected from the dam (Latschar et al., '49) at the same time. This occurred not only in cows receiving no tocopherols but also in those supplemented with this vitamin at the 0.5–1 gm level.

The data from one newborn calf indicated rapid absorption of the colostral tocopherols ingested. At birth, the tocopherol concentration per 100 ml of blood serum was 56 μg, but three hours after the feeding of 2.25 kg of colostrum containing 3.46 mg of tocopherols per 100 ml the level had raised to 111 μg, a 100% increase. Six hours after feeding the level was 105 μg.

Serum tocopherols in some calves from unsupplemented dams increased somewhat from the 3rd to 14th days after birth. Though this appears to be a normal change which is somewhat different than that found for vitamin A and carotenoids (Wise et al., '48), the trend might have been affected in some instances by small amounts of colostrum included in the milk fed. The average serum tocopherols in all groups of calves had decreased appreciably by the 28th day. Although considerable individual variation within the groups was still apparent at this age, the tocopherol levels of normal calves from dams that received supplements of this vitamin were within the same range as those from dams that received no tocopherol supplements. Decreases in the levels of serum tocopherols were observed when calves were ill; note the results for calves from dams G-432, G-451, J-358 and J-365 (table 2).

TABLE 2

Tocopherol concentrations in the blood serum of calves [1] from dams receiving rations either unsupplemented or supplemented with tocopherols during late stages of gestation

PREPARTAL VITAMIN SUPPLEMENT OF DAM [2]	DAM OF CALF	DAILY INTERVALS AFTER BIRTH							
		0 [3]	1	2	3	7	14	21	28
		tocopherols, μg/100 ml							
	H-105 [4]	56	124	167	155	229	378	260	260
	H-127	12	105	161	210				
	H-175	105	204	167	161	266	272	223	99
None	H-219	25	155	173	272				
	G-432	43	192	248	149 [5]	254	297	223	105
	G-433	43	173	136 [6]	266	198	279	194	254
	Mean	47	159	175	202	237	307	225	180
None [7]	H-126	25	68	62	80				
	H-115	25	111	87	74	186	285	161	118
	H-128	68	149	161	173				
Vitamin A	H-137	0	118	180	217				
alcohol or	J-358	31	130	68 [6]	118	167	173	124 [8]	50 [8]
ester	J-365	68	161	192	229	310	204 [6]	285	254
	G-465	68	186	297	291				
	G-468	25	62	206	272				
	Mean	40	131	178	196	221	221	190	141
Vitamin A	H-135	136	217	347	328				
alcohol plus	H-136	74	229	322	285				
tocopherols, 0.5–1 gm	Mean	105	223	335	304				
Tocopherols, 0.5–1.0 gm	G-451	43	334	260 [6]	229 [6]	192	297	291	192
Tocopherols, 4 gm mixed	G-467	68	520	588	520	198	260	186	105
Tocopherols, 10 gm mixed	A-200	87	241	464	285				
	J-356	93	501	730	619	706	415	210	155
	Mean	90	371	597	450				

[1] Calves (table 1) that were continued on trials beyond first day of life. Each calf received colostrum from its own dam for first three days; see text for ration after third day.

[2] See table 1 for kinds and rates of vitamin supplements given prepartally.

[3] Before colostrum ingestion.

[4] Letters H, A, J and G refer to Holstein, Ayrshire, Jersey and Guernsey, respectively.

[5] Calf refused part of feed previous P.M.

[6] Calf diarrhetic.

[7] Dam milked throughout gestation.

[8] Calf ill.

The changes observed in the serum tocopherols of calves during the first three days might be expected to be related to the tocopherol content of the colostrum ingested. Since there was considerable variation in the tocopherol concentrations in the serum of calves at birth and in the colostrum from cows of the same experimental groups, the extent of this relationship is not apparent from the data of table 2. As the tocopherol content of the colostrum fed the calves increased, serum tocopherols similarly increased (table 3); but differences in tocopherol levels in the serum did not parallel those in the colostrum ingested. Even when the data are arranged as in table 3, a considerable degree of individual variation in response is still evident.

TABLE 3

Relation of tocopherol concentrations in colostrum ingested to concentrations in blood serum of calves

NUMBER OF CALVES IN GROUP [1]	TOCOPHEROL CONC. IN DAILY FEEDINGS OF COLOSTRUM [2]			INCREASES IN SERUM TOCOPHEROLS OVER LEVELS AT BIRTH		
	Days after birth			Days after birth		
	0 [3]	1	2	1	2	3
	μg/100 ml			*μg/100 ml*		
2	230	133	96	65	50	52
	(198–262) [4]	(124–142)	(77–114)	(43–86)	(37–62)	(49–55)
7	569	372	174	92	162	152
	(398–717)	(177–619)	(92–260)	(37–155)	(93–248)	(56–247)
4	818	380	178	117	182 [5]	181 [5]
	(777–876)	(263–563)	(108–291)	(68–149)	(111–229)	(99–223)
1	1,520	511	256	291	217 [6]	186
3	3,069	1,609	1,037	338	511	392
	(2,630–3,820)	(1,348–2,045)	(663–1,780)	(154–452)	(377–637)	(198–526)

[1] Calves grouped according to the average tocopherol concentration of colostrum ingested the first two feedings after birth.

[2] Colostrum fed at a daily rate of 1 lb. of body weight of the calf.

[3] Values given for day zero are averages for first two postnatal feedings.

[4] Range of values.

[5] Results from one diarrhetic calf eliminated.

[6] Calf diarrhetic.

DISCUSSION

It appears from the foregoing results that the immediate postnatal mammary secretions are more effective than prenatal transmission in the endowment of newborn calves with serum tocopherols. This is in agreement with changes observed in vitamin E in newborn rats (Mason and Bryan, '40). However, in addition to the tocopherols found in the serum, the newborn apparently obtain additional quantities through placental transfer, since the livers of lambs, kids and pigs were found to contain stores of this vitamin (Whiting and Loosli, '48). Prepartal tocopherol supplementation of the dam in experiments by these workers did not increase significantly the concentrations of this vitamin in the livers of the newborn.

In the present study, serum tocopherol levels in newborn calves from unsupplemented dams were found to be higher than those reported in the plasma of lambs and kids by Whiting and Loosli ('48), but the rations fed to pregnant ewes and does by the latter workers were deficient in tocopherol activity. These investigators found that, as compared to the newborn of unsupplemented animals, tocopherol levels in the plasma of lambs and kids were increased by prepartal tocopherol supplementation of the dam. Under the conditions of the present study with calves, smaller increases resulted from prepartal supplementation of the dam.

The relative quantitative importance of placental and mammary transfer of serum tocopherols, the rapid changes upon ingestion of colostrum, and the trends during early postnatal life resemble previous findings with respect to vitamin A (Wise et al., '48; Spielman et al., '49). Serum tocopherol levels at birth and the levels attained after colostrum and milk feeding at the various stages are about 10-fold [4] those observed for vitamin A. Although, as compared to vitamin A, relatively high levels of tocopherols were found at birth, and colostrum ingestion greatly amplified the serum reserves of this vitamin in the newborn, the tocopherol requirements

[4] Seven-fold on a molar basis.

of the calf for normal development during early life have not been established (Gullickson et al., '44). Furthermore, it was found in studies with 6 calves (Wise and Murley, '48) that supplementing normal rations with tocopherols from the 4th to 64th day of life improved neither general health nor growth.

SUMMARY

A study was made of serum tocopherol levels in calves, at birth and during the first month of life. The calves were from cows fed either typical unsupplemented barn rations or similar rations supplemented during the terminal stages of gestation with vitamin A or tocopherols, or both. The levels of supplemental tocopherol fed the dams were 0.5–1, 4, 5 and 10 gm daily. The first food the calves received was colostrum from their respective dams, followed later by a whole milk and grain-roughage diet.

Supplements fed the dams prepartally did not markedly affect the serum tocopherol levels in their newborn calves. Tocopherols per 100 ml of serum were 44 ± 31 μg in calves from unsupplemented cows and only 79 ± 20 μg in those from cows supplemented at the highest level.

Calves born with low serum tocopherol levels seemed to be as vigorous and healthy at birth as did those having the highest levels.

The serum tocopherol levels of newborn calves increased three- to 9-fold following the ingestion of colostrum. The highest levels were found in calves whose dams received large amounts of supplemental tocopherols, but considerable individual variation in response was noted.

By the 28th day after birth, tocopherol concentrations in calves were lower than during the period of colostrum feeding, and values for calves from supplemented cows were within the same range as those for calves from the unsupplemented.

Decreases in serum tocopherol levels were observed in calves that were diarrhetic.

ACKNOWLEDGMENTS

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DENTAL CARIES IN THE COTTON RAT

XI. THE EFFECT OF FEEDING A NATURAL DIET COMPARABLE TO A HUMAN DIET [1]

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Earlier experiments in our laboratory have been primarily concerned with the effect of various synthetic diets on dental caries' production in the cotton rat. These purified diets are not comparable with the human diets which are known to produce extensive dental disease in man. Therefore a study was made of dental caries' development in cotton rats receiving a diet resembling the average human diet. The specific effect upon dental disease in the cotton rat of varying some of the components of this natural diet is reported.

EXPERIMENTAL

Cotton rats from our stock colony were put on experiment at weaning and kept on the experimental rations for 14 weeks. To minimize the effect of strain variation in caries susceptibility, as described by Schweigert et al. ('45), animals from each litter were distributed equally between control and experimental groups. At the end of the experimental period the animals were sacrificed and the incidence and extent of

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dental caries evaluated according to the method of Shaw et al. ('44).

The selection of a representative human diet which the cotton rats could eat was a difficult task. The diets given by Lane et al. ('42), based on the annual per capita consumption of foods as estimated by Stiebling and Phipard ('39), were used as a basis for the selection of the experimental diets.

All of the foods in these diets were dehydrated and ground in a Wiley mill until they passed through a medium-fine screen. The necessity of having a dry uniform diet that would resemble in physical characteristics our synthetic ration demanded a severe simplification of the diet suggested by Lane et al. The composition of the experimental diets is given in table 1. In all of the experiments the diets were fed ad libitum. All animals received two drops of haliver oil each week.

In experiment 1, in addition to natural diet 834 with 17% sucrose, diets 835 and 836 containing 32.3 and 47.3% sucrose, respectively, were fed to cotton rats to study the effect upon dental caries of increasing the amount of refined sugar in a diet of natural foodstuffs. In both diets the additional sucrose was added at the expense of the cereals and bread portion of the rations.

In experiment 2 we studied the effect on dental disease in cotton rats of reducing the amount of refined sugar in the natural diet. Diet 834, after being checked against the synthetic control diet 802, was used as the control diet in this experiment. The sucrose was reduced in diets 854 and 856 to 0% and 2%, respectively, and coarse dextrin, a previously demonstrated cariogenically inert substance, was used to replace the sucrose in these diets. In this experiment an additional group of rats was given natural diet 855 containing 17.3% sucrose but with the dried milk portion of the diet replaced by dextrin. These animals were then given liquid whole milk ad libitum as the only source of fluid.

The bread component of the diet was investigated more thoroughly in experiment 3 by substituting 36% enriched

TABLE 1

The percentage composition of the diets fed to the cotton rat

INGREDIENTS	DIET									
	802	825	851	852	834	835	836	854	856	855
Sucrose	67	16.75	16.75	16.75	17.3	32.3	47.3	0	2.0	17.3
Dextrin		50.25	14.25	47.25				17.3	15.3	14.6
Casein	24	24	24	12						
Corn oil	5	5	5	5	3.4	3.4	3.4	3.4	3.4	3.4
Salts IV	4	4	4	4						
Wheat gluten protein				12						
Cereal and bread										
Ralston					2.9	1.7	0.5	2.9	2.9	2.9
Rolled oats					2.9	1.7	0.5	2.9	2.9	2.9
Bread (white enriched)			36		30.0	17.3	4.8	30.0	30.0	30.0
Dried whole milk					14.6	14.6	14.6	14.6	14.6	0
Meat										
Corned beef hash					9.4	9.4	9.4	9.4	9.4	9.4
Dehydrated pork and beef					6.6	6.6	6.6	6.6	6.6	6.6
Vegetables										
Potatoes					7.1	7.1	7.1	7.1	7.1	7.1
Cabbage					5.6	5.6	5.6	5.6	5.6	5.6

white bread for part of the dextrin in the otherwise synthetic diet number 825, which had previously been shown by Schweigert et al. ('46a) to be only moderately cariogenic. The protein of the bread was studied by substituting wheat gluten for one-half of the protein in synthetic diet 825.

RESULTS

Natural diet 834 was tolerated very well by the cotton rats. The animals on this diet consumed more food and made better gains than their litter-mate controls on the synthetic cariogenic diet, 802.

TABLE 2

Effects of increasing the level of sucrose in a natural diet on the incidence and extent of carious lesions

RATION FED	NUMBER OF ANIMALS	CARIOUS LESIONS	
		Average incidence	Average extent[1]
802 (Cariogenic diet)	4	28	80 +
834 (Natural diet containing 17.3% sucrose)	4	27	80 +
802 (Cariogenic diet)	4	31	87 +
835 (Natural diet with 32% sucrose)	4	25	76 +
802 (Cariogenic diet)	4	30	87 +
836 (Natural diet with 47% sucrose)	4	33	115 +

[1] Individual lesions are arbitrarily evaluated 1 + to 5 +.

The dental caries data expressed as averages of incidence and of extent are given in tables 2, 3 and 4. In all cases the average caries scores of the experimental groups are compared only with the average caries scores of their litter-mate controls.

Natural diet 834, which contained only 17.3% refined carbohydrate as sucrose, produced as many and as severe dental caries in the cotton rat as were produced by the feeding of cariogenic synthetic diet 802 with 67% sucrose. Increasing the level of sucrose to 32% in natural diet 835 did not increase the number or severity of the dental lesions. However, there

was an increase in the severity of the dental lesions in the animals receiving natural diet 836 containing 47% sucrose.

Cotton rats which were fed the natural diet without any sucrose, diet 854, had almost complete protection from dental caries. Replacing 2% sucrose (diet 856) increased the caries scores appreciably when compared with the scores of animals receiving no sucrose at all. However, the caries scores were

TABLE 3

Effects of decreasing the level of sucrose in a natural diet on the incidence and extent of carious lesions

RATION FED	NUMBER OF ANIMALS	CARIOUS LESIONS	
		Average incidence	Average extent [1]
802 (Cariogenic diet)	3	18	40 +
834 (Natural diet — 17% sucrose)	3	14	35 +
834 (Natural diet — 17% sucrose)	10	15	40 +
854 (Natural diet — 0% sucrose)	10	3	5 +
834 (Natural diet — 17% sucrose)	5	17	44 +
856 (Natural diet — 2% sucrose)	5	13	16 +
834 (Natural diet — 17% sucrose)	5	17	45 +
855 (Natural diet with dried whole milk plus liquid whole milk)	5	13	32 +

[1] See footnote, table 2.

TABLE 4

The effect of bread and wheat gluten on the production of dental caries

RATION FED	NUMBER OF ANIMALS	CARIOUS LESIONS	
		Average incidence	Average extent [1]
802 (Cariogenic diet)	5	26 ± 3	68 +
825 (¾ dextrin, ¼ sucrose)	5	9 ± 5	19 +
825 (¾ dextrin, ¼ sucrose)	9	9 ± 5	14 +
851 (36% bread in 825)	9	14 ± 6	26 +
825 (¾ dextrin, ¼ sucrose)	7	16 ± 6	31 +
852 (825 with ½ protein as wheat gluten)	7	15 ± 7	31 +

[1] See footnote, table 2.

still much lower in the animals receiving the natural diet containing 2% sucrose than in those receiving the original diet with 17.3% sucrose.

When bread was substituted for dextrin in moderately cariogenic synthetic diet 825 at a level of 36% there was no significant increase in the production of dental caries. The data in table 4 indicate an increased caries incidence when bread was fed. However, when the individual animal scores were examined such overlapping of data was found as to make this increase appear insignificant. Substituting wheat gluten for 50% of the total protein in synthetic diet 825 gave diet 852 which, when fed to cotton rats, resulted in the same caries scores as were found in animals receiving control diet 825. No protection from dental caries was noted in animals receiving liquid whole milk separately along with ad libitum feeding of natural diet 855 (i.e., with dried milk left out).

DISCUSSION

Previous studies by Shaw et al. ('44) and Schweigert et al. ('46a, b) have shown that the production of dental caries in the cotton rat is associated with the presence of fermentable carbohydrate in the diet. The data reported here lend further support to this theory, since of the dietary constituents studied the omission of only sucrose from the natural diet gave nearly complete protection from dental caries in the cotton rat. However, it must be noted that simplified natural diet 834 containing only 17.3% refined sugar (the amount eaten by the average American) was capable of producing as severe dental disease in cotton rats as the synthetic diet with 67% sucrose. Furthermore, increasing the sucrose level of the natural diet to 32.3 or 47.3% did not appreciably affect the caries' production. There are several possible explanations for these results. They may indicate that these animals have a carbohydrate tolerance level within which few, if any, caries are produced and over which rampant caries develop regardless of the amount of sugar added in excess of this limit.

Although sucrose comprised only 17.3% of the basic experimental diet, the cereal, bread, dried whole milk, corned beef hash and potatoes of the diet contributed carbohydrate to raise the total level to about 50%. Cereals have received considerable attention in the past as caries-promoting foods (Larsen, '49; Hoppert et al., '32; Belding and Belding, '39). Therefore it is quite probable that the 6% of cereal in this diet, which was of the acid residue type, may have contributed to the cariogenicity of the diet. The dried whole milk of the diet contributed 5.5% lactose, a carbohydrate which has been shown by Schweigert et al. ('46a) to have cariogenic properties.

The substitution of bread for dextrin in a moderately cariogenic diet fed to cotton rats did not increase dental disease, but this does not completely prove that the bread did not contribute to caries' production in the rats fed the natural diet because the physical state of the two diets was different. The particles of the natural diet were small and uniform but they varied in hardness. Some of the dried food particles, particularly those of the dried potato, were very hard and probably produced fracture. It has been shown by Bibby and Sedwick ('33) that many of the cavities in the teeth of white rats receiving a coarse and hard particle diet originated as fractures.

The feeding of liquid milk in addition to the dry natural diet did not reduce dental disease. This is in contrast to the work of Anderson et al. ('47), in which they observed reduction in caries in cotton rats which received liquid milk in addition to a dry synthetic diet. However, Anderson and associates restricted the amount of dry ration given to the rats and the animals were forced to drink more of the liquid milk.

Although natural diet 834 had a moderately high fat level, it produced severe dental caries in the animals receiving it. Schweigert et al. ('46a) report that the feeding of a medium

fat synthetic diet to cotton rats gave protection from dental decay. The variance in physical state of the two diets may have contributed to this difference in results.

The effect of the adequacy of a diet upon dental caries' production, which has been given support by the researches of various investigators, cannot be completely disregarded as a factor in this experiment. However, the cotton rats receiving our natural diet made gains as good as or better than those made by their litter-mate controls receiving a synthetic diet fortified with minerals and vitamins. Furthermore, in this natural diet the white bread, which made up 36% of the ration, was enriched with minerals and vitamins and thus contributed to the adequacy of the diet.

SUMMARY

A natural diet containing foods found frequently in the human diet and only 17% sucrose, the proportion consumed by the average person in the United States, produced as many and as severe carious lesions in the cotton rat as did a basal cariogenic diet with 67% sucrose. Increasing the sucrose level in the diet to 32% or 47% at the expense of the cereal and bread portion did not appreciably affect the incidence or extent of caries. Reducing the sucrose level of the diet to 0% or 2% reduced the occurrence of caries by 80% and 60,% respectively.

When the 14.6% dry whole milk was omitted from the diet and liquid whole milk was given separately as the animals' only source of fluid, the same caries scores were obtained as were obtained when natural diet 834 was fed ad libitum without liquid milk.

Substitution of 36% white bread for dextrin in a moderately cariogenic diet did not appreciably affect the cariogenicity of this ration when it was fed to cotton rats.

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TRYPTOPHAN DEFICIENCY IN THE RAT INDUCED BY FORCED FEEDING OF AN ACID HYDROLYZED CASEIN DIET

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EIGHT FIGURES

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Investigations with diets deficient in an essential amino acid are complicated by the fact that test animals eat very little of such a diet. The amount consumed usually permits very little growth and may even be insufficient for maintenance of the body weight of pair-fed control animals receiving an adequate supplement of the missing amino acid. The publications of Maun, Cahill and Davis ('45a, b, '46), Albanese et al. ('43), and Harris et al. ('43) may be cited in this connection.

When tryptophan-deficient diets were allowed ad libitum, a minimum of 50 days was required to produce anemia in young rats and a longer period in older rats (Albanese et al., '43). By contrast, one of the present authors, using a forced-feeding technique in a study of the metabolic interrelationship among tryptophan, pyridoxine and nicotinic acid in the rat (Spector, '48), found that all animals (24) receiving no tryptophan died in from 6 to 13 days. One rat was sacrificed for histological examination on the 10th day of the experiment. The liver was light colored, and the

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hepatic cells were swollen and highly vacuolated, suggesting fatty infiltration.

Forced feeding is a useful technique in nutrition studies because it not only completely eliminates complications produced by generalized undernutrition, but it also accentuates the inherent effect of any dietary modification. It has the added advantage of decreasing the time required to produce the syndrome resulting from the dietary modification (Spector, '48).

The present paper reports the changes induced in rats by forced feeding of a tryptophan-deficient diet and includes a brief summary of the histological observations.[2]

EXPERIMENTAL PROCEDURE

Young male and female rats of the Sprague-Dawley strain were fed variable amounts of ground laboratory chow [3] until the weight of each animal was about 100 gm at 6 weeks of age. In addition, 4 mature rats, 18 weeks of age, were also studied. The acid-hydrolyzed casein diet similar to that on which tryptophan deficiency was previously produced (Spector, '48) had the following percentage composition: acid-hydrolyzed casein 20, vitamin-free casein [4] 2, mineral mixture 5, corn oil 3, DL-methionine 0.6, and sucrose 69.4. All vitamins were supplied in adequate amounts.[5] The tryptophan content of this ration was only 0.024%, all coming from the vitamin-free casein. The deficient diet was supplemented with 0.20% L-tryptophan for the controls. Both the control and the deficient diet were force-fed in equal amounts three times daily

[2] The detailed histological findings will be presented elsewhere in a separate report.

[3] Purina.

[4] Labco.

[5] The following vitamins were supplied, in milligrams per 100 gm of diet: thiamine 0.25, riboflavin 0.50, calcium pantothenate 2.0, choline chloride 100, inositol 10, *p*-aminobenzoic acid 50, biotin 0.01, folic acid 0.025, 2-methylnaphthoquinone 0.1; and halibut liver oil (diluted 1:2 with corn oil) at a level of two drops per week, with α-tocopherol included at 0.5 mg per drop.

by the technique previously described (Spector, '48). The food intake was gradually increased from 7.5 to 12 gm per day.

The original plan of the experiment was to sacrifice one male and one female from the group on the deficient diet on alternate days over a period of three weeks. One male and female on the control diet were to be sacrificed on the 7th, 14th and 21st day. Due to circumstances beyond control and the death of some of the experimental animals, it was not possible to follow this schedule. The actual treatment and distribution of the 35 experimental animals was as follows: (1) tryptophan-deficient, young 19, mature 3 (18 weeks); (2) control (0.20% L-tryptophan), young 6, mature 1; (3) curative response to 7 days of control diet after 10 days on tryptophan-deficient diet 4; (4) stock [6] 2.

After the selected rats were weighed and blood was taken from the tails for hemoglobin determinations, they were killed by decapitation without anesthesia. Selected organs were immediately removed and weighed. Tissue samples were then fixed in preparation for histological study and frozen sections of liver tissue were prepared for study of the fat distribution. The remaining livers (major part) and carcasses were frozen for later chemical analysis. Several rats on the tryptophan-deficient diet died before they could be sacrificed for histological study, and none lived longer than 16 days.

The dry matter (70°C. in a vacuum oven), fat (ether extract), and nitrogen determinations were done according to the official A.O.A.C. methods.

EXPERIMENTAL RESULTS

The appearance and behavior of the tryptophan-deficient animals are partially pictured in plate 1, which also permits a comparison with a control animal (fig. 3). Figures 5–8 were selected from 35 mm motion picture frames showing typical behavior and postures observed in the deficient rats. The

[6] See footnote 3, page 214.

hunchbacked position is typical of these animals but is probably not peculiar to tryptophan deficiency (Albanese and Buschke, '42; Ferraro and Roizin, '47). A general alopecia, rough haircoat, and diarrhea contribute to the unkempt appearance of the deficient rats.

As previously noted (Spector, '48), the tryptophan-deficient animals displayed the following peculiar behavior pattern after forced feeding: (1) pawing the face as though trying to withdraw the food; (2) crawling around the edge of the cage with the side of the face resting on the floor of the cage; (3) rearing up on the hind legs and pawing frantically with the front paws; (4) after 5 to 10 minutes they would go to sleep, with a tendency to sleep on the side. The latter may have been due to the fact that these rats were frequently bloated and the abdominal area was sensitive. Figure 8 shows a common posture which the deficient animals assumed, presumably to favor the sensitive bloated abdominal area. The stomach and intestines were found at autopsy to be greatly distended and filled with liquid and gas.

The rats developed a nervous hyperexcitability which commonly manifested itself in convulsive fits accompanied by screeching, during which they bounced helplessly against all the sides of the cage. These fits frequently ended in death. The behavior of the tryptophan-deficient rats resembled in a general way that of the valine-deficient rats observed by Rose and Eppstein ('39).

Due to complications introduced by diarrhea and bloating, the changes in weight of the deficient animals are best considered in general terms. The over-all range in weight change of the deficient animals was -16 to $+21$ gm. The control animals gained on the average 20 gm in 7 days and a total of 47 gm in the 23 days of the experiment. The four "curative responsive" animals all lost 8 to 16 gm (average 12) in the 10 days on the deficient diet and gained 15 to 18 gm (average 17) in the 7 days on the tryptophan-supplemented diet.

Due to the highly suggestive picture of fatty infiltration of the liver observed in a previous study (Spector, '48), the con-

tent and distribution of fat in the liver were thoroughly investigated both by chemical and histological means. A detailed scattergram of the per cent of fat in the liver (dry matter basis) is presented in figure 1.

Increase in the concentration of fat in the liver is an apparent trend in the deficient animals. From levels of 4.8 and 6.3% on the second day the concentration of fat rapidly increased to 40.1 and 46.5% on the 7th and 9th days, respectively. This is very high for a diet containing 3% corn oil as the only fat.

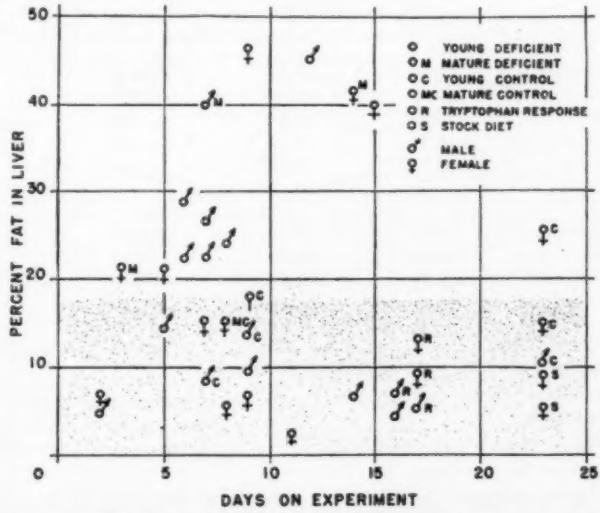

Fig. 1 The effect of tryptophan on the per cent of fat in the liver (dry matter basis). The shaded area indicates the zone of normal values.

There was considerable unexplainable variability in the liver fat concentration, and several deficient animals had values below 10% even up to the 16th day. But the prevalence of functional variability in biological studies, even under conditions as well controlled as possible, has long been widely recognized. Although the average fat concentration in the livers of the deficient animals (20.84%) was about 30% more

than the average of the controls (15.34%, table 1), the coefficient of variation in the deficient group was so large (68%) that the differences were not statistically significant. The decreased dry matter content (fat-free basis) of the livers of the deficient animals was not statistically significant. The reduction in the nitrogen concentration (dry matter, fat-free basis)

TABLE 1

The average dry matter, fat and nitrogen concentration of the livers and carcasses

	TRYPTOPHAN DEFICIENT	TRYPTOPHAN SUPPLEMENTED	DIFFERENCE [1]	P [2]
	%	%		
		Liver		
Dry matter [3]	26.45	28.76	−2.31	0.15
Fat [4]	20.84	15.34	+5.50	0.34
Nitrogen [5]	11.23	13.05	−1.82	< 0.001
Nitrogen [5]	11.23	13.17 [R]	−1.94	0.008
Nitrogen [5]	11.39 [Td]	13.79 [Ts]	−2.40	0.001
		Carcass		
Dry matter [3]	27.14	25.76	+1.38	0.07
Nitrogen [6]	12.95	13.16	−0.21	0.22
Fat [4]	25.29	31.98	−6.69	0.01
Fat [4]	25.29	21.99 [R]	+3.30	0.31
Fat [4]	31.53 [Td]	33.55 [Ts]	−2.02	0.30

[1] Difference from tryptophan-supplemented values.

[2] Probability of chance outcome.

[3] Fat-free basis.

[4] Dry matter basis.

[5] Dry matter, fat-free basis.

[R] Tryptophan response after 10 days on the deficient diet.

[T] Terminal animals: deficient = 12–16 days; supplemented = 23 days.

was very highly significant ($P = < 0.001$). This was confirmed by the return to normal of the "response" animals and by comparison of the values for the three final controls (23 days) and 5 "terminal" deficient animals (12 to 16 days).

In the chemical analysis of the carcasses no significant differences were found in the dry matter and nitrogen concentrations. The total average concentration of fat in the carcass was statistically significantly less for the tryptophan-deficient

animals than for the controls. The "response" animals, however, had an even lower carcass fat concentration than the deficient animals. Further doubt is cast upon the actual significance of the differences in fat concentration of the carcasses when the values are compared with those for the "terminal animals" ($P = 0.3$). There was a general increase in the fat concentration of the carcass in all groups during the experiment as a whole. The apparent significant difference in total averages is probably explained by the fact that

TABLE 2

The average organ weight expressed as a percentage of the body weight

	TRYPTOPHAN DEFICIENT	TRYPTOPHAN SUPPLEMENTED		DIFFERENCE [1]	P [2]
		Control	*Response* [3]		
Liver	7.01	4.74		+ 2.27	< 0.001
	7.01		4.71	+ 2.30	0.007
Spleen	0.183	0.310		— 0.127	< 0.001
	0.183		0.373	— 0.190	0.008
Pancreas	0.568	0.664		— 0.096	0.49
	0.568		0.740	— 0.172	0.25
Kidney	0.604	0.514		+ 0.090	< 0.001
	0.604		0.573	+ 0.031	0.30

[1] Difference from tryptophan-supplemented values.
[2] Probability of chance outcome.
[3] Tryptophan response after 10 days on the deficient diet.

the deficient average includes more values from the early days of the experiment when the values were lower.

The weight of each organ removed was compared with the body weight and expressed as a percentage of the latter. The 4 organs listed in table 2 were the only ones which showed any definite change. The liver and the kidney hypertrophied, while the spleen and the pancreas atrophied. The liver increased 47.8% in the deficient animals. Due to the large variation encountered, the decrease in the size of the pancreas is not significant. The changes in the liver and spleen were

reversible, as is seen in the percentage weights of the "response" animals.

A reduction in the hemoglobin level of the tryptophan-deficient animals was first observed on the 9th day (fig. 2). The average hemoglobin content of the blood of three deficient animals at 14 to 15 days was only 11.6 gm per 100 ml, while that of the 4 control animals was 14.0. This reduction in hemoglobin concentration was statistically significant ($P = 0.01$). Two of the "response" animals showed a return to normal

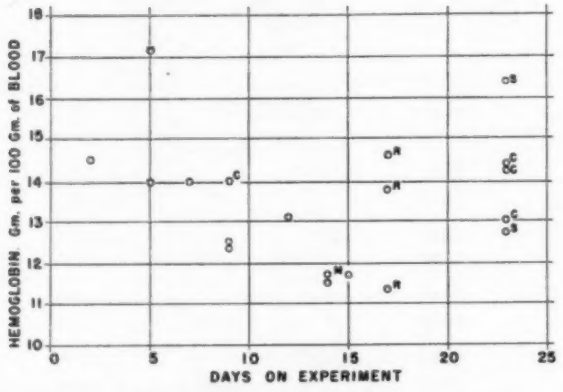

Fig. 2 The effect of tryptophan on the hemoglobin concentration. (The same symbols are used as in fig. 1.)

levels (14.6 and 13.8), while one remained at the low level of 11.25. This same "response" animal had developed an unusually large spleen and also failed to show the decrease in weight of the liver associated with "cure."

HISTOLOGICAL OBSERVATIONS

The histological study of the tissues, which is presented in detail in another report, showed that most of the organs were normal. However, in the tissues described below some striking abnormalities were observed in the tryptophan-deficient rats.

As the deficiency of tryptophan increased, the livers of the deficient animals showed progressive accumulations of fat in the hepatic cells. This condition was cured when the deficient animals were given an adequate supplement of L-tryptophan. The fatty infiltration was accompanied by the appearance of a single large nucleolus within the nucleus of the liver cells, in contrast to the occurrence of several nucleoli in the normal nucleus. The mitochondria also became larger and vesicular but less numerous.

The testes of the deficient animals showed degenerative changes in various stages of germ cell formation and in some cases sloughing of the germinal epithelium occurred. This condition suggests ultimate sterility if the degenerative process were allowed to continue.

The smooth muscle of the wall of the intestine and urinary bladder as well as the cardiac muscle showed indications of degenerative changes. Injury to epithelial tissue was limited to sloughing of the lining of the urinary bladder in a few rare cases and to keratinization of the cornea of the eye in more advanced cases of tryptophan deficiency.

DISCUSSION

In the sense that certain protein fractions of the body, such as those of blood plasma and of the liver, can be readily broken down to supply nitrogen for other purposes one can speak of a protein reserve. As Madden and Whipple ('40) have expressed it, "A part of the body protein forms a reserve against adversity in the sense that it can be circumspectly depleted without apparent injury to the body." In proportion to the original content the liver loses the most protein (40%) of any tissue in the albino rat during a 7-day fast (Addis, Poo and Lew, '36).

In the present studies the liver lipids of the tryptophan-deficient rats seemed to increase progressively. These findings are consistent with the observations of Seifter et al. ('48) that rats on a protein-free diet showed an increase in liver fat and a decrease in liver nitrogen with the changes proportional

to the duration of the depletion period. There was no significant loss of nitrogen from the body of the rats force-fed a tryptophan-deficient diet. Addis, Poo and Lew ('36) found that only 8% of the original protein of the muscle, skin and skeleton was lost in rats during a 7-day fast. Furthermore, forced feeding of the tryptophan-deficient diet, by satisfying the caloric maintenance requirements, probably conserved tissue proteins for their specific functions (Cannon, '48).

The livers of rats on a non-protein diet for 8 to 18 days were observed by Miller ('48) to show a loss of activity of the enzymes studied. The recent work of Lehninger and Kennedy ('48) has definitely established that the fatty acid oxidase activity of the liver and the system of enzymes involved in the Krebs tricarboxylic acid cycle are located exclusively in the mitochondria of the rat liver cell as the mitochondria are separated by differential centrifugation methods. It may be significant that the mitochondria in the cells of the fatty livers of the tryptophan-deficient animals were unlike those of the control animals in size, shape and number, which may indicate some interference with the mechanism of fat metabolism in the liver. The normal lipid concentration and nearly normal histologic picture of fat distribution seen in the livers of tryptophan "curative response" animals were paralleled by an almost normal appearance of the mitochondria. This might explain the reversibility of the fatty accumulation in the liver seen in the tryptophan-deficient animals.

Fatty infiltration of the liver has not been reported in a deficiency of any other single amino acid. The liver was moderately atrophied in leucine deprivation (Maun et al., '45b) and normal (or not studied) in deficiencies of the other amino acids. The increase in size and fatty infiltration of the liver may, therefore, be characteristic of tryptophan deficiency.

The effects of deficiencies of the 10 essential amino acids in the various mammalian species studied thus far are summarized in a chart by Follis ('48). One is struck by the paucity of information available. Follis has been critical in his review of the literature and has refused to list the non-

specific changes that were observed as a result of inanition. Harris et al. ('43) have remarked that "the whole picture (of lysine deficiency) is not unlike that produced by ordinary starvation." Histological examinations of the tissues of rats deficient in histidine (Maun, Cahill and Davis, '46) and in phenylalanine (Maun et al., '45a) showed no changes other than those which can be ascribed to inanition.

The tissue protein synthesizing mechanism is "perfectionistic" (Cannon, '48), inasmuch as all the essential amino acids which are to enter into the process must be simultaneously available. Thus, a deficiency of any essential amino acid hampers effective protein synthesis, whether for growth or maintenance, which results in a general picture of deficiency common to all essential amino acids. It is difficult, therefore, to establish the specificity of pathological changes peculiar to the deficiency of a particular amino acid as distinguished from the general picture of deficiency common to all essential amino acids. In the following discussion an effort will be made to ascertain the specificity of the changes observed in tryptophan deficiency.

In the tryptophan deficiency studies of Albanese et al. ('43), a minimum of 58 days was required to produce a slight anemia in young rats, and for mature rats at least 86 days were required. Furthermore, two young rats with initial weights of 108 and 109 gm survived 107 days on a tryptophan-deficient diet, at which time the hemoglobin was 11.9 and 12.1 gm %, respectively. When tryptophan deficiency was induced in the present investigation by forced feeding, the hemoglobin was reduced to about 11.5 gm % in both young and mature (170 gm) rats in only 14 days. Also, in the present study, as previously, the tryptophan-deficient animals did not survive beyond 16 days. However, all of the essential amino acids have been found to be necessary for hemoglobin and plasma protein formation (Maun, Cahill and Davis, '46; Madden and co-workers, '40, '45). Corneal vascularization and lenticular changes of some degree have also been observed in deficiency of protein and of each of the indispensable amino acids except

arginine (Hall et al., '48). The depletion of the vitamin A stores of rats, the decrease in their serum proteins, and the reduction of their capacity to form antibodies when immunized with typhoid vaccine observed by McCoord et al. ('47) are probably non-specific effects of the general derangement of protein metabolism brought about by the absence of tryptophan in the diet.

Alopecia is a characteristic feature of tryptophan deficiency in the rat (Totter and Day, '42; Albanese and Buschke, '42; Spector, '48). Although loss of hair occurs in deficiencies of zinc, riboflavin, pantothenic acid, biotin and inositol (Follis, '48), it has not been reported as a consistent result in a deficiency of any other amino acid.

The male germinal epithelium is extremely sensitive to inanition. Consequently, testicular atrophy is a prominent part of many nutritional deficiencies (Follis, '48). In the investigations of Maun, Cahill and Davis ('45a, b, '46), degeneration and atrophy of the semeniferous tubules of the testes were found in rats deprived either of phenylalanine or of leucine, and the spermatogenesis of the histidine-deficient rats was delayed. Holt et al. ('42) have reported a marked reduction in the number of spermatozoa of three men after 9 days on an arginine-deficient diet, although nitrogen equilibrium was maintained throughout this period. Albanese and Buschke ('42) refer to the observation of atrophy of the testis and aspermiogenesis in rats on a tryptophan-deficient diet, but no details are given.

The peculiar behavior pattern of the tryptophan-deficient rats has now been observed in two series of experiments. Aside from the sensitivity to touch, the lack of coordination in movement, and a tendency to rotate in a circular manner observed in a deficiency of valine (Rose and Eppstein, '39; Ferraro and Roizin, '47), no other amino acids have produced similar deficiency syndromes in rats.

While necrosis of myocardial fibers has been found as a result of both potassium and thiamine deficiencies and scar-

ring of the myocardium as a result of vitamin E deficiency, no lesions have as yet been described in the smooth muscle of mammals deficient in one or more of the essential nutrients, according to Follis ('48). Atrophy and degeneration of both the cardiac and smooth muscle fibers were observed in the rats in the present experiments who were force-fed the tryptophan-deficient diet.

The spleen was reported to atrophy moderately in leucine deficiency but was normal in deficiencies of phenylalanine and histidine (Maun, Cahill and Davis, '45a, b, '46). In our tryptophan-deficient rats there was an average reduction of 40% in the size of the spleen in relation to body size. Therapeutic supplementation with tryptophan produced a return to normal size, with definite evidence of over-compensation. The kidneys hypertrophied, perhaps due to the extra load of amino acids they were forced to handle because of the poor utilization of the protein associated with deficiency of tryptophan and the high level at which the protein was maintained because of forced feeding. However, both the spleen and kidney were found to be normal on histological investigation.

In the present experiment the mature animals were not distinguishable from the young in their susceptibility to the changes produced by tryptophan deficiency induced by forced feeding of the deficient diet. Apart from the degenerative changes found in the semeniferous tubules of the testis, males and females showed the same effects of tryptophan deficiency. No essential differences were observed between the control animals force-fed the tryptophan-supplemented diet and the animals receiving a stock diet ad libitum.

SUMMARY AND CONCLUSIONS

Young and mature rats of both sexes of the Sprague-Dawley strain were force-fed three times daily an acid-hydrolyzed casein diet deficient in tryptophan. The deficient diet was supplemented with 0.20% L-tryptophan for the con-

trol animals. Rats from both groups were sacrificed for chemical and histological examination at regular intervals to determine the progressive changes produced by tryptophan deficiency.

The tryptophan-deficient rats exhibited a general alopecia, bloating and diarrhea. A peculiar behavior pattern was commonly observed after forced feeding, sometimes accompanied by convulsive fits which frequently ended in death.

There were no significant changes in the dry matter, fat and nitrogen concentrations of the carcass. There was enlargement of the kidney, while the spleen atrophied. There was marked hypertrophy of the liver with a reduction in the nitrogen concentration, although the fat-free dry matter content was not significantly changed. A slight anemia was produced, which was also cured by tryptophan. There was degeneration of the semeniferous tubules in the testes of male rats.

The previous finding of fatty infiltration of the liver was confirmed by both chemical analysis and histological examination, and, because the mitochondria in the cells of fatty livers were less numerous and different in size and shape from those of the control animals, it is probable that the fatty accumulation is a result of decreased fatty acid oxidase activity. This system of enzymes of the liver is located exclusively in the mitochondria. The changes in percentage weight of the liver and spleen were reversible, as was the fatty infiltration of the liver when curative treatment was initiated after 10 days on the deficient diet.

Atrophy and degeneration of visceral and cardiac muscle are striking effects of tryptophan deficiency. Injury to epithelial tissues occurred in a few organs, but its importance appears limited. Keratinization of the cornea was recognizable after about 9 days of tryptophan deficiency and was usually accompanied by other ocular lesions, which have been adequately described by Albanese and Buschke ('42).

ACKNOWLEDGMENTS

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PLATE 1

EXPLANATION OF FIGURES

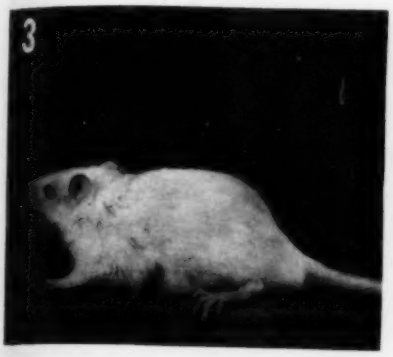
3 Control rat (0.20% L-tryptophan).

4 Tryptophan-deficient diet.

5–8 Enlargements from 35 mm motion picture frames of tryptophan-deficient rats.

5 and 8 Show two variations of the hunchbacked posture.

7 Shows the pawing of the face while standing up on hind legs.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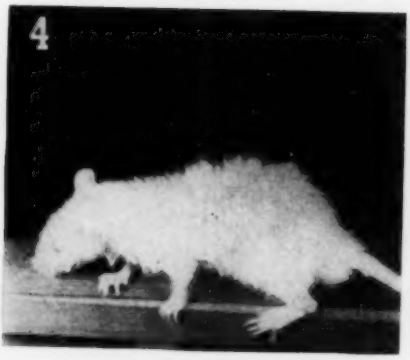
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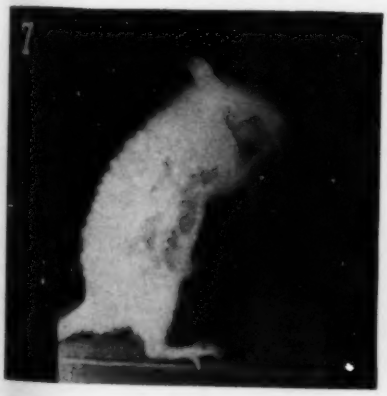
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AN EVALUATION OF THE PROTEIN QUALITIES OF SIX PARTIALLY PURIFIED PROTEINS

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Six protein samples were submitted to this laboratory by the Bureau of Biological Research of Rutgers University for evaluation as to their relative protein qualities. Samples of these same proteins were also distributed to several other laboratories for similar analysis by various chemical and biological methods. The results compiled from these studies are to be used by the Rutgers group in an effort to resolve some of the discrepancies observed in the literature regarding protein quality. The evaluations presented in the present report are based on growth and protein efficiency data obtained by feeding the samples to rats at a 1.44% nitrogen level.

The following protein samples were received in a dry powdered form and therefore required no further processing for the test: egg albumin, whole egg powder, beef muscle, casein, wheat gluten and peanut flour.

EXPERIMENTAL

Since 4 separate feeding tests were carried out, using two different rations, the tests and data will be presented individually.

Test 1

Six groups of weanling male rats (8 rats per group) were fed 6 different rations containing the test proteins at a

1.44% nitrogen level (dry weight). The animals[1] were housed in individual cages, and were kept in a room in which the temperature and humidity were carefully controlled. The diets were prepared on an isocaloric basis with respect to fat and protein (table 1) and were fed ad libitum, with fresh diet being supplied two or three times a week. During

TABLE 1

Percentage composition of the diets used in test 1

INGREDIENTS	DIETS					
	A	B	C	D	E	F
Dextrose	15	15	15	15	15	15
Salt mixture[1]	4	4	4	4	4	4
A and D oil (3,000 I.U. A, 400 I.U. D/gm)	1	1	1	1	1	1
Wheat germ oil	1	1	1	1	1	1
Vitamin mixture[2]	0.71	0.71	0.71	0.71	0.71	0.71
Choline chloride	0.1	0.1	0.1	0.1	0.1	0.1
Lard	14.96	14.28	14.9	14.97	14.98	14.97
Starch	53.64	49.01	52.50	52.91	51.81	51.57
Beef muscle	9.59	....	....	....	....	....
Peanut flour	....	14.90	....	....	....	....
Wheat gluten	....	....	10.79	....	....	....
Casein	....	....	....	10.31	....	....
Egg albumin[3]	....	....	....	....	11.40	....
Whole egg powder	....	....	....	....	....	11.65

[1] Jones and Foster salt mixture (J. Nutrition, *24:* 245; 1942).

[2] Thiamine 0.8 mg, riboflavin 0.8 mg, pyridoxine hydrochloride 0.8 mg, Ca pantothenate 4.4 mg, niacin 4.0 mg, 2-methyl-1,4 naphthoquinone 1.0 mg, liver 1:20 powder 0.7 gm.

[3] An aqueous supplement of 25 μg of biotin was given each rat per day, 5 days a week.

the 8-week experimental period, the rats were weighed at weekly intervals and an accurate record of the amount of food consumed was maintained. From the growth and food consumption data, protein efficiency values were calculated, and these are presented, together with other pertinent data, in table 2.

[1] Obtained from the Holtzman Rat Company.

TABLE 2

Growth, food consumption and protein efficiency data

	DIET	NO. OF WEEKS	AVE. TOTAL WT. GAIN PER RAT	STANDARD ERROR OF MEANS	AVE. TOTAL FOOD USED PER RAT	AVE. TOTAL N USED PER RAT	GAIN PER GM N CONSUMED	STANDARD ERROR OF MEANS
			gm.		*gm.*	*gm.*	*gm.*	
Test 1	A Beef muscle	8	111	(7.1)	487	7.01	15.83	(0.68)
	B Peanut flour [1]		40	(4.5)	366	5.27	7.59	(0.46)
	C Wheat gluten [2]		3	(1.2)	231	3.33	00.90	(0.41)
	D Casein		95	(10.0)	437	6.29	15.10	(0.58)
	E Egg albumin		154	(11.1)	517	7.44	20.70	(0.64)
	F Whole egg		94	(6.9)	451	6.49	14.48	(0.49)
Test 2	G Beef muscle	8	117	(4.6)	520	7.49	16.96	(0.73)
	H Peanut flour		49	(3.3)	389	5.60	8.75	(0.37)
	I Wheat gluten		14	(0.9)	243	3.50	4.00	(0.29)
	J Casein		136	(6.2)	564	8.12	16.75	(0.42)
	K Egg albumin		220	(8.7)	666	9.59	22.94	(0.58)
	L Whole egg		166	(8.0)	647	9.32	17.81	(0.60)
	M Lactalbumin		166	(7.9)	566	8.15	20.37	(0.51)
Test 3	N Lab. beef muscle no. 1	8	205	(5.4)	726	10.45	19.61	(0.28)
	O Lab. beef muscle no. 2		201	(4.6)	668	9.62	20.89	(0.35)
	G Rutgers beef muscle		147	(3.4)	632	9.10	16.15	(0.33)
	P Raw round beef [3]		168	(4.1)	490	7.06	23.79	(0.65)
	Q Raw loin beef		180	(10.6)	518	7.46	24.13	(0.82)
	M Lactalbumin		175	(2.8)	603	8.68	20.16	(0.37)
Test 4	N Lab. beef muscle no. 1	5	125	(3.1)	461	6.64	18.82	(0.74)
	O Lab. beef muscle no. 2		126	(3.2)	431	6.21	20.29	(0.24)
	G Rutgers beef muscle		87	(2.8)	395	5.69	15.29	(0.43)

[1] One animal dead (cause of death unknown).

[2] Two animals dead (cause of death unknown).

[3] One animal dead (respiratory infection).

Test 2

This test was carried out in a manner analogous to that of test 1 except for several modifications of the basal diet. The compositions of the rations used are given in table 3. Growth and food consumption data were collected for the 8-week experimental period and are summarized, together with protein efficiency calculations, in table 2.

Since beef muscle gave relatively poor results in both tests, it was decided to compare this preparation with several others made in our laboratory (test 3).[2]

[2] *Preparation of Rutgers beef muscle sample.* The entire carcass of a utility grade beef was used. The meat was trimmed free of visible fat and gristle and then ground. It was heated to 180°F., spread on trays, and dried in vacuum from the frozen state. The dried preparation was defatted with benzol and again dried in vacuum at low temperature to remove all solvent. The resulting product was ground sufficiently fine for incorporation into diets.

Preparation of laboratory beef muscle preparation no. 1. A sample of the insides (top round) and outsides (bottom round) of utility grade round beef was trimmed free of all visible fat and ground. This material was allowed to stand in water for approximately one-half hour and the supernatant decanted and discarded. The ground muscle residue was resuspended in water and the process of extracting and decanting continued until the muscle appeared white and the supernatant became colorless. The muscle was then pressed free of water and suspended in an excess of alcohol (10% methyl in 95% ethyl alcohol). The suspension was stirred frequently and allowed to stand overnight. This procedure was repeated three times. The cake was washed three times with diethyl ether and allowed to stand overnight each time. The cake was pressed nearly free of ether and air dried.

Preparation of laboratory beef muscle preparation no. 2. A sample of the insides (top round) and outsides (bottom round) of utility grade round beef was trimmed free of all visible fat, sliced and cooked with agitation in an equal amount of water at 175° ± 10°F. for one hour. The cold meat was added to boiling water. The broth was drained off and discarded. The cooked meat was ground and dehydrated in a hot air drier. The maximum temperature reached was not over 210°F., and that only momentarily at the end of the period. Total drying time was 60 to 70 minutes. The dehydrated product was ground and extracted with hexane at a low temperature to a low fat content. The beef preparation was air dried in a vacuum chamber with a final drying period of 8 hours.

TABLE 3

Percentage composition of the diets used in tests 2, 3 and 4

INGREDIENTS	DIETS										
	G	H	I	J	K	L	M	N	O	P	Q
Salt mixture [1]	4	4	4	4	4	4	4	4	4	4	4
A and D oil	1	1	1	1	1	1	1	1	1	1	1
Wheat germ oil	1	1	1	1	1	1	1	1	1	1	1
Vitamin mixture [2]	0.71	0.71	0.71	0.71	0.71	0.71	0.71	0.71	0.71	0.71	0.71
Choline chloride	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Agar	2	2	2	2	2	2	2	2	2	2	2
Lard	9.96	9.28	9.9	9.97	9.98	9.96	9.57	9.63	9.91	9.73	9.99
Dextrose	71.64	67.01	70.5	70.91	69.81	69.58	71.06	71.93	70.56	70.54	70.31
Beef muscle	9.59	....	....	....	....	....	....	....	....	....	....
Peanut flour	....	14.90	....	....	....	....	....	....	....	....	....
Wheat gluten	....	....	10.79	....	....	....	....	....	....	....	....
Casein	....	....	....	10.31	....	....	....	....	....	....	....
Egg albumin	....	....	....	....	11.40	....	....	....	....	....	....
Whole egg powder	....	....	....	....	....	11.65	....	....	....	....	....
Lactalbumin [3]	....	....	....	....	....	....	10.56	....	....	....	....
Lab. beef prep. no. 1	....	....	....	....	....	....	....	9.63	....	....	....
Lab. beef prep. no. 2	....	....	....	....	....	....	....	....	10.72	....	....
Raw round of beef	....	....	....	....	....	....	....	....	....	39.50	....
Raw loin of beef	....	....	....	....	....	....	....	....	....	....	44.0

[1] Jones and Foster salt mixture (J. Nutrition, *24:* 245; 1942).

[2] The same vitamin mixture (test 1) was used in these diets except for the addition of 200 μg % of biotin.

[3] Swift's commercial lactalbumin.

Test 3

For this test, two preparations of beef muscle were made by different methods using the insides (top round) and outsides (bottom round) of utility grade beef. The methods by which these preparations were made are given in footnote 2. They were ground in a Wiley mill to a fine powder and were fed at a 1.44% nitrogen level, as in the case of the other proteins. Similarly, two samples of raw beef, one utility grade insides and outsides of round beef and the other ufility grade loin of beef, were trimmed free of all visible fat and ground. Care was exercised in handling the meat to insure a minimum of contamination. The diets containing the samples were prepared in the same manner as the foregoing and sufficient raw beef was added to give 1.44% nitrogen on a dry weight basis. It was assumed that the rats would consume a sufficiently greater quantity of diet to compensate for the added moisture. The two raw beef diets were divided into small portions of 150 to 200 gm, wrapped individually in wax paper and stored at $-10°$F. They were fed fresh daily, all uneaten food being dried, reweighed and discarded. All other diets were fed fresh two or three times a week and the unconsumed food reweighed. The basal diet used in these studies was similar to that used in test 2 (table 3). As in the foregoing tests, growth, food consumption and protein efficiency values were collected for the 8-week experimental period and are presented in table 2.

Test 4

The data obtained from test 3 indicated that the beef muscle preparation supplied by Rutgers University was of a significantly inferior quality when compared to the two samples prepared in our laboratory. To make certain of the reproducibility of these results, the test was repeated, using freshly prepared diets and employing the same procedure as before. The data obtained in this experiment are also summarized in table 2.

DISCUSSION

A statistical analysis of the data was made and the standard errors of the means [3] for each group in each test are given in table 2. Where the comparisons drawn were important, t values were also calculated and may be found in the text. The formula used may be expressed as $t = (\bar{x}_1 - \bar{x}_2)/\sqrt{s^2_1 + s^2_2}$, where the subscripts refer to the two groups under consideration.

A comparison of the results obtained in tests 1 and 2 reveals that better performance was obtained with the ration used in test 2. With regard to the relative protein qualities of the 6 proteins, both tests demonstrate that egg albumin supplemented with biotin is of superior protein quality. In test 1, beef muscle gave the next highest value, followed by whole egg powder and casein which gave statistically the same value. In test 2, whole egg powder gave the second best value, followed by casein and beef muscle which were both significantly inferior. Either the replacement of starch with dextrose or the addition of biotin may have been responsible for the slightly different but superior results obtained in test 2. Peanut flour and wheat gluten gave very poor results in both tests.

The relative qualities of the 6 proteins as determined by the methods and conditions employed in our laboratory agree essentially with those expressed in the literature for similar samples of protein. Biological values for samples of the same 6 proteins were determined with human patients by Hawley et al. ('48) and were found to be of the following order: beef muscle 67, peanut flour 56, wheat gluten 42, casein 56–68, egg albumin 91 and whole egg powder 94. Chow et al. ('48) determined the nutritive values of 5 proteins by the protein efficiency method of Osborne and Mendel ('26) and by the Cannon depletion method ('45). The protein values were found to occur in the following order: egg white, lactal-

[3] The formula used for calculating the standard error for each mean may be given as: $s = \sqrt{\Sigma (x - \bar{x})\, 2/n(n-1)}$, when x is an item observed and n is the number of such items involved.

bumin, whole egg powder, casein and wheat gluten. This same order did not necessarily follow, however, in plasma protein regeneration studies with dogs. Allison et al. ('49) found the nitrogen balance indexes of raw dried egg white, raw dried whole egg protein, casein and wheat gluten, when fed to normal dogs, to be 1.12, 0.88, 0.74 and 0.4, respectively. When fed to protein depleted dogs, these indexes were 1.12, 1.06, 0.84 and 0.70, respectively. Comparisons of the relative biological values of a series of proteins with dried whole egg taken as a standard were reported by Mitchell and Block ('46). These values were found to be beef muscle 76, casein 73, egg albumin 82, lactalbumin 84 and peanut flour 58. Digestibility of all samples ranged between 97 and 100%.

One item of especial interest to us was the relatively poor performance of the Rutgers beef muscle preparation when compared to several samples of beef muscle prepared in our laboratory (tests 3 and 4). Even though the value which we obtained for the Rutgers sample agrees with that reported by a number of investigators, still other workers have nevertheless reported a higher value for beef. For example, Hoagland and Snider ('46) demonstrated an average growth promoting value of 19.7 gm gain per gram of beef nitrogen consumed, as compared to 26.8 gm gain for rats receiving dried egg, when diets contained 10% protein. Later, Hoagland et al. ('48) reported an average gain of 21.9 gm per gram of beef muscle nitrogen and 24.9 gm gain per gram of egg protein nitrogen consumed when both proteins were fed at a 10% level. From the data presented in table 2, an average protein efficiency value of 16.05 gm gain per gram of nitrogen consumed can be assigned to the Rutgers beef preparation as determined in 4 separate feeding tests. The two laboratory preparations of beef muscle, on the other hand, gave respectively in two tests an average gain of 19.21 and 20.59 gm per gram of nitrogen consumed. Therefore it would appear that the Rutgers sample was of inferior quality

when compared to the two samples of beef protein prepared in our laboratory.[4]

It is difficult to suggest a reason for this discrepancy, since gentle treatment was exercised in the preparation of the Rutgers sample (see footnote 2, page 234). One essential difference may be that the Rutgers sample was made using the entire beef carcass, whereas we used specific sections of the carcass. In test 3 (table 2), however, no significant differences in quality could be observed between samples of raw round of beef and loin of beef. There remain the differences in the source of material and in the methods used in handling and preparing the meat for test. In preparing our materials a certain amount of extraction was carried out in which some nitrogen was undoubtedly removed, whereas the preparation of the Rutgers sample was limited to extraction with benzol.

The fact that the animals consuming the raw beef diets failed to gain as well as those receiving the laboratory preparations may possibly be explained by the fact that the rats receiving the raw diets did not consume as much food on a dry weight basis. The rather high moisture content of the diet (approximately 30%) may have limited the food intake. Since their food conversion was significantly better than that of the groups receiving the laboratory beef preparations, these rats might have grown better than the other groups had they consumed more food.

[4] A statistical treatment of the data obtained in tests 3 and 4 yielded the following conclusions:

Test no.	Comparison	*t* value for ave. total wt. gain	*t* value for gm gain per gram N_2 consumed
3	Rutgers beef vs. lab. beef prep. 1	8.98	7.65
3	Rutgers beef vs. lab. beef prep. 2	9.51	7.19
4	Rutgers beef vs. lab. prep. beef 1	9.16	3.74
4	Rutgers beef vs. lab. beef prep. 2	8.78	5.40

SUMMARY

Six protein samples distributed for collaborative study were evaluated by the rat growth method for their relative protein qualities, and were found to be in the following order in these respects: egg albumin, whole egg powder, beef muscle, casein, peanut flour and wheat gluten.

The beef muscle preparation supplied for these studies may not have been representative of beef since it gave markedly inferior results when compared to two preparations of beef muscle made in our laboratory and to two samples of raw beef.

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ADDENDUM

The following pertinent statement was furnished by the committee in charge of the Rutgers Cooperative Study of Protein Evaluation:

"The cooperative study of 6 protein foods coordinated by the Rutgers Bureau of Biological Research was undertaken in September 1946 for the following purposes: (1) to compare the several methods of determining the nutritive value of protein foods in normal and protein-depleted animals; (2) to determine the differences in such evaluation which are dependent upon (a) the species of animals used, and (b) the quality of the protein fed; (3) to select, if possible, one method for evaluation of a given protein for preliminary standardization purposes; and (4) to compare the several methods for determining the amino acid content of the protein foods. These purposes do not include a study of the effects of processing or sampling.

"Four hundred pounds of each protein in dry powdered form were prepared by one manufacturer, and delivered to the Bureau in one thoroughly mixed lot for distribution to the cooperating investigators. The necessary amino acid standards for assay were assembled, repurified where necessary, and characterized by one manufacturer, and delivered to the Bureau for distribution.

"The proteins were selected chiefly on the bases of their ranging from excellent to poor, and of their present and future availability in large amounts. Certain proteins which might have been selected upon theoretical grounds were thereby eliminated. It was fully recognized that some of the proteins would be altered from their natural state by the processing necessary to prepare them in one large batch for a two-year program. Such alterations, however, should not interfere with the study's purposes.

"Results obtained by the 20 evaluating and 12 assay laboratories were reported in full to the Bureau, which in turn will distribute a complete summary to each investigator and sponsor, and to all others interested. Each investigator retained full publication rights for his own work. The Bureau is planning to publish a summary of the program."

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REQUIREMENT OF THE PIG FOR VITAMIN B_{12}

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TWO FIGURES

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Earlier work in this field was reviewed by Hogan and Anderson ('48). After the experimental work in that paper was completed, vitamin B_{12} was isolated (Rickes, Brink, Koniuszy, Wood and Folkers, '48) and was shown to be essential for the chick (Ott, Rickes and Wood, '48). Our studies on the requirement of the pig for this vitamin have been described in a brief abstract by Hogan and Anderson ('49), and similar observations were reported by Neumann, James, Krider and Johnson ('49). The latter workers used a soybean protein in their experimental diet as a source of nitrogen, and this material may be contaminated with vitamin B_{12} to a lesser degree than is casein.

EXPERIMENTAL

The experimental procedure used has been described by McRoberts and Hogan ('44) and Hogan and Anderson ('48), and only its more essential features will be repeated. The newborn pigs were left with the sow for two days, as in our earlier experience the mortality rate had been high, even on diets believed to be adequate, if the pigs failed to receive colostrum. The diets described in table 1 were synthetic in type but homogenized in water to a solids content of 19%. The pigs were hand-fed from a graduated cylinder fitted with a rubber

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nipple at three-hour intervals, beginning at 6:00 A.M. and ending at 12:00 midnight. All of the pigs were females and they were confined in cages with wide-mesh wire floors in a room held at a temperature of 75°F. or higher. Four of the pigs were pure-bred Chester Whites and 4 were Chester White-Duroc Jersey crossbreds.

TABLE 1

Composition of experimental diets

INGREDIENT	RATION 327	RATION 350	VITAMIN SUPPLEMENT PER 100 GM OF DIET [1]	
	%	%		
Casein, vitamin-free [2]	30	20	Thiamine-HCl	1 mg
Sucrose	30	65	Riboflavin	1 mg
Cornstarch	5	..	Pyridoxine-HCl	1 mg
Lard	30	5	Ca-pantothenate	3 mg
Salt mixture [3]	5	5	Nicotinic acid	4 mg
Wood pulp	..	5	Choline chloride	100 mg
			Biotin	0.03 mg
			Inositol	100 mg
			Pteroylglutamic acid	0.2 mg
			Vitamin E	4 mg
			Vitamin K	2 mg
			Vitamin A	2,000 I.U.
			Vitamin D	400 I.U.

[1] Vitamins A and D were purchased as a concentrate from Mead Johnson and Company, Evansville, Indiana. The pteroylglutamic acid was generously supplied by Dr. T. H. Jukes, Lederle Laboratories Division, American Cyanamid Co., Pearl River, New York. All other vitamins were generously supplied by Dr. D. F. Green of Merck and Company, Inc., Rahway, New Jersey.

[2] Labco, purchased from the Borden Company, New York, N. Y.

[3] Richardson and Hogan ('46).

The experimental diets, described in table 1, were of the conventional synthetic type. The casein was described as vitamin-free but unfortunately, according to microbiological assay, it contained 1.7 μg % of vitamin B_{12}. Six pigs were divided into two groups. Each pig in one group received intramuscular injections of crystalline vitamin B_{12} [2] at three-day intervals,

[2] The vitamin B_{12} was generously supplied by Dr. D. F. Green of Merck and Company, Inc., Rahway, New Jersey.

beginning when they were two and ending when they were 38 days of age. The total amount injected in that period was 50 μg for one pig, 100 μg for the second, and 200 for the third. When the experimental pigs were 10 weeks old they were placed in pens with concrete floors, bedded with wood shavings, and the experimental diet was supplied in dry form, with no added water. Because of wastage the food intake could not be measured accurately after this time and no records of food intake were made. When the pigs were 17 weeks old the diet was changed to no. 350. Two pigs, used as positive

TABLE 2

Administration of vitamin B_{12} and individual weights of pigs

AGE IN DAYS	PIG NO.					
	113	174	171	173	114	170
	Amount of vitamin B_{12} in micrograms					
2–8	6	12	24			
11–20	12	24	48			
23–26	8	16	32			
29–38	24	48	96			
88–114				25		
	Weights of pigs in kilograms					
56	15.9	13.6	14.8	12.1	13.5	13.0
147	65.8	73.9	76.2	39.5	[1]	59.4

[1] Dead.

controls, were supplied with cow's milk that had been fortified with 60 gm sucrose, 2.5 gm ferrous sulphate, 0.2 gm cupric sulphate, 0.2 gm manganous sulphate, and 0.02 gm of potassium iodide, per liter. These two animals were discarded at 8 weeks of age.

Intermittent attacks of diarrhea during the first 8 weeks were observed in all three pigs that did not receive vitamin B_{12} injections, and they were especially severe in pigs 114 and 170 (table 2). These two pigs, especially no. 114, vomited frequently after being fed, and the amounts consumed at successive feedings were often highly variable. Pig 113 received the

smallest amount of vitamin B_{12}, and she developed a severe case of diarrhea and loss of appetite when she was 16 weeks old but recovered within three weeks. The first indications of recovery became apparent the week after the ration was changed to no. 350, but it was uncertain whether or not the change was responsible for the improvement. The three pigs that were given vitamin B_{12} injections also had mild diarrhea during the first two weeks but, with the exception noted, they were comparatively free from this disorder thereafter.

During the first 6 weeks all 6 pigs that consumed the synthetic diet grew at a uniform rate, which was about the same as that called for by currently used growth standards. The three that had been given the vitamin B_{12} injections were not appreciably heavier than their controls, though it is possible that their haircoats were sleeker and glossier. After the first 6 weeks, however, the pigs that had received the injections began to outstrip the controls, and by the 20th week they were growing at a tremendous rate. The three pigs that received the vitamin B_{12} injections were continued on the experimental diet after the others were discarded, in an attempt to determine whether the vitamin is essential during gestation and lactation, but it will be several months before these studies are completed.

As was mentioned previously, diet 327 was supplied with no added water after the 10th week. The pigs that had received the vitamin B_{12} injections adapted themselves to the change within a few days and then continued to grow at the expected rate. The pigs that had not received the injections, however, were slow in becoming adjusted. During the two weeks before the change was made pig 114 vomited occasionally and had a mild diarrhea and an erratic appetite. This animal died soon after the diet was supplied in dry form, and post mortem examination revealed congestion and a fatty metamorphosis in the liver and extreme congestion and necrosis in the kidneys. Acute pneumonia was the immediate cause of death.

Pig 173 had a persistent loss of appetite, began to chew on the wooden pen partitions, and ate large quantities of the shavings supplied for bedding. In order to prevent the eating of shavings she was again placed in a cage with wide-mesh screen floors. A mild exudate appeared on her back and along her sides, with localized skin eruptions. Eighteen days after the form of the diet was changed, when the pig was 88 days old, she had lost 3 kg in weight and death seemed imminent. It appeared probable that her condition was due to a deficiency of vitamin B_{12}, and she was given a total of 25 μg of this vitamin by intramuscular injection, divided into three doses over

Fig. 1 Pig 173. Age 119 days; weight 15.9 kg. Scattered mild lesions of the skin developed, then disappeared shortly after the administration of vitamin B_{12}.

a period of 26 days. The first injection was administered when she was 88 days old and the third and last was administered 26 days later. She made a complete recovery after the last injection, gained rapidly in weight, improved in appearance, gave no sign of an abnormal appetite, and the skin lesions shown in figure 1 disappeared. This pig was killed for examination when 147 days of age, but no abnormalities were revealed by gross inspection. Histological examination indicated that the heart, spleen and muscle were normal but a few small areas in the kidney were hyperemic and a somewhat greater degree of abnormality was observed in the liver; the hepatic cells were granular and there was some evidence of fatty in-

filtration. It is possible that insufficient time had elapsed for complete recovery in this organ. The appearance of the pig, before the vitamin B_{12} injections were started and after recovery, is shown in figure 2. The third pig in this group lost weight during the 11th week, after the form of the diet was changed, but she recovered and thereafter grew at a rate that exceeded current growth standards. She was discarded at the age of 147 days.

The amount of vitamin B_{12} required by the pig is of considerable importance, but our data on this point are incomplete. Complicating factors are the amount of vitamin B_{12}

Fig. 2 Pig 173. A. Age 87 days; weight 10.9 kg. B. Age 147 days; weight 39.5 kg. In the interval between 88 and 114 days of age this pig received 25 μg of vitamin B_{12} by intramuscular injection. The skin lesions are disappearing.

that was stored in the tissues at the beginning of the experimental period and the amount that adhered to the casein as a contaminant. Furthermore, the vitamin was supplied orally in the casein consumed and by intramuscular injection at the same time, and there is no reliable conversion factor that would permit a calculation of the total requirement by either mode of administration. However, it seems desirable to make the best estimates that the data now available will permit, though there is no pretense that any of them has a firm experimental basis. Spies, Lopez, Milanes, Toca and Aramburu ('49) used vitamin B_{12} that had been incubated with human

gastric juice for the treatment of pernicious anemia. When administered orally the required dosage was 5 to 10 times higher than when it was administered by parenteral injection. However, one cannot take it for granted that this ratio would apply to simple deficiency in the pig.

Nichol, Dietrich, Cravens and Elvehjem ('49) have published data on chicks which can be applied to this problem, though they are much too scanty to permit any degree of precision in reaching conclusions. One might conclude from their report that a daily intramuscular injection of 0.1 μg of vitamin B_{12} gave about the same gain in weight as did the addition of 1 μg to each 100 gm of diet. If one chick ate an average of 20 gm of food each day, 0.1 μg of vitamin B_{12} given by injection would equal 0.2 μg administered orally. One could assume on the basis of a report of Johnson and Neumann ('49) that 0.1 μg of vitamin B_{12} administered to the pig by injection is the equivalent of 0.25 μg administered orally. The findings in these reports are in excellent agreement, under the circumstances. We have applied the 1:2 ratio of Nichol et al. to the portion of our data which seems most suitable for the purpose, obtained with pig 113. This animal lost her appetite temporarily when about 88 days old, and it is assumed that the amount of the vitamin she received, in the diet and by injection, was enough to last her for 84 days. The available data are collected below.

Average weight to 84 days	12.5	kg
Feed consumed	34	kg
Vitamin B_{12} in 100 gm of casein	1.7	μg
Vitamin B_{12} in the feed consumed	174	μg
Equivalent of 174 μg given orally, when given by intramuscular injection	87	μg
Vitamin B_{12} by intramuscular injection	50	μg
Injected intramuscularly + equivalent of amount in the feed	137	μg
Equivalent of 50 μg injected intramuscularly when administered orally	100	μg
Supplied orally + equivalent of amount given by intramuscular injection	274	μg
Kilogram — days	1,050	

(1) Amount of vitamiin B_{12} required daily per kilogram of live weight:
a. When administered by intramuscular injection
137 ÷ 1,050 ≏ ..... 0.13 μg
b. When supplied orally
274 ÷ 1,050 ..... 0.26 μg
(2) Amount of vitamin B_{12} required per 100 gm of ration:
a. Estimated from the total quantity administered
274 ÷ 340 ≏ ..... 0.8 μg
b. Estimated from the amount of vitamin B_{12} in the basal diet.
The diet contained approximately 0.5 μg of vitamin B_{12} per 100 gm, and some of the pigs developed deficiency symptoms. One might suppose, then, that 1 μg per 100 gm would meet the minimum requirement and that 1.5 μ would be more than enough to maintain an optimum nutritional state.

Few published data are available that would supply evidence of the reliability of the estimates given above. One could conclude that the diet supplied to chicks by Ott, Rickes and Wood ('48) became adequate when 1.5 μg of vitamin B_{12} per 100 gm of diet were included. According to our calculations, their basal diet probably contained 0.35 μg per 100 gm, giving a total of 1.85 μg. It is not yet possible to decide whether that amount is insufficient or excessive, or whether it should be the recommended allowance. Neumann and collaborators ('49) stated in a brief abstract that 4.2 μg of vitamin B_{12} per 100 gm of dry matter in the diet is sufficient for the maximum growth of pigs and for hematopoiesis.

It is necessary to make some assumptions in applying the data of Johnson and Neumann ('49) for our purpose, but we estimate that their pigs received 0.46 μg of vitamin B_{12} by injection for each 100 gm of food consumed. On the basis of their 1:2.5 ratio this would be the equivalent of 1.15 μg administered orally. Data on the amount of vitamin B_{12} adhering to the isolated soybean protein are not available and the deficiency symptoms described would indicate that it is small. However, the report of Neumann, Krider and Johnson ('48) indicates that isolated soybean protein contains almost as much vitamin B_{12} as does "vitamin-free" casein. If it does

contain that much, it would supply about 0.5 μg per 100 gm of food, or 1.65 μg in all. For the present we assume that the diet of the pig need supply not more than 1.5 μg per 100 gm of ration.

The response of the pigs that were supplied with cow's milk deserves some comment. By the time they were two

TABLE 3

Average food consumption and gains in weight by experimental animals

RATION NO.	327 B_{12}		327				COW'S MILK	
	No. of pigs							
	3		1		2		2	
Age	Wt.	Feed consumed	Wt.	Feed consumed	Wt.	Feed consumed	Wt.	Feed consumed
days	*kg*	*kg*	*kg*	*kg*	*kg*	*kg*	*kg*	*kg*
0	1.1		1.1		1.4		1.2	
7	1.8	0.305	1.8	0.311	2.1	0.363	2.0	0.441
14	2.3	0.527	2.4	0.510	2.7	0.564	2.9	0.788
21	3.6	1.003	3.5	0.862	4.0	0.992	4.4	1.009
28	5.1	1.267	4.8	1.148	5.1	1.046	6.6	1.703
35	6.9	1.677	6.5	1.294	6.9	1.451	9.1	2.284
42	9.0	2.197	8.2	1.980	9.0	2.072	13.0	4.015
49	11.2	2.842	10.0	1.997	10.9	1.966	18.1	5.040
56	14.8	3.353	12.1	2.257	13.3	2.372	22.9	5.483
63	17.9	3.785	13.5	2.192	15.3	2.743		
70	21.0	4.325	14.3	2.300	16.7	2.520		
84	27.0		11.8		17.1			
119	48.8		15.9 [1]		35.6			
147	72.0		39.5		59.4			

[1] The decline in weight (pig 173) was arrested at this point by an injection of vitamin B_{12}, as described in the text and in table 2.

weeks old they were definitely heavier than the experimental animals, and by the time they were 8 weeks old they weighed over 50% more than the experimental animals. They ate more food but why they ate more, and gained more, is a question of some importance. It may be that the experimental diet, because of physical texture or chemical composition, is irritating to the alimentary tract of a young pig, or it may have been partially inadequate. Our data on vitamin dosage and on

the individual weights of the pigs are shown in table 2. More complete data on the average weights and food consumption are summarized in table 3.

SUMMARY

Young pigs were reared on a synthetic diet and given intramuscular injections of vitamin B_{12}. There was no definite response until the pigs were 6 weeks old. After that time they grew at an unusually rapid rate.

One of the control pigs died. Another became emaciated and developed an abnormal appetite, followed by mild lesions of the skin. After the injection of 25 μg of vitamin B_{12} she recovered rapidly. The third pig in this group developed no deficiency symptoms and grew at the normal rate.

The tentative estimate of the quantitative requirement for vitamin B_{12} when administered orally is 0.26 μg daily per kilogram of live weight, or not over 1.5 μg per 100 gm of food.

Two pigs supplied with fortified cow's milk grew more rapidly than did those that received the vitamin injections. It is suggested that an unrecognized vitamin is essential for the optimum nutritional state.

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A STUDY ON THE RELATION AND ADJUSTMENT OF BLOOD PLASMA LEVEL AND URINARY EXCRETION OF ASCORBIC ACID TO INTAKE

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INTRODUCTION

Human retention of ascorbic acid as based on maintained blood plasma ascorbic acid levels on regularly increasing amounts of ascorbic acid, in the range of intake found in the ordinary dietary, has been reported for college women by this laboratory (Dodds and MacLeod, '44). Weekly plasma values were shown to be a better indication of individual intake than single values, and a retention of approximately 1 mg of ascorbic acid per kilogram of body weight was indicated by limited urinary excretion data. The detailed data of this and other studies made in this laboratory were submitted to the Institute of Statistics of the University of North Carolina with a view to investigating the possibilities of greater economy in the use of time and subjects and of increasing the significance of findings with regard to blood plasma and urine adjustments to ascorbic acid intake.

On the basis of the analysis of these data it was found that large variability existed between individuals as compared to the variability within individuals, and that more information could be gained by studying more subjects and obtaining

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fewer individual records. Two consecutive daily blood values were shown to be efficient for indicating response to intake. Judging by successive 4-day averages, a delay of about 4 days in reacting to an increased intake of ascorbic acid occurred, and the full effect of the 25-mg increase used in the experiment was usually reached within two weeks. Investigation of adjustment to intakes has been a special objective in this laboratory and intakes have been restricted to the amounts ordinarily encountered rather than being enlarged to comprise saturation doses.

EXPERIMENTAL

Based on these observations, the following plan was used in succeeding studies: Three intakes of ascorbic acid — 50, 75, and 100 mg — were selected. These were augmented by the amount in the diet (6.9 ± 2.68 mg), which was analyzed by the Loeffler and Ponting method ('42). In this paper intakes refer to supplements of synthetic ascorbic acid. Four experiments were conducted, two in the winter in January and February (average temperature 58°F.), and two in the summer in June and July (average temperature 78°F.). The experimental design was set up as two 3×3 Latin Squares, orthogonal to each other (table 1).

Six subjects were used in each experiment. They were college women between the ages of 19 and 29 and were considered in good physical condition as judged by medical examination. The average weight was 56.8 kg with a range of from 45.7 to 74.3 kg, and none was overweight. All 6 subjects received the three intakes but the sequence differed for each. Thus increases and decreases of 25 and 50 mg of ascorbic acid intake were studied and adjustment times followed. A preliminary period of 6 days on a 25-mg ascorbic acid intake preceded the three experimental periods of 12 days each. Blood plasma ascorbic acid analyses by the Mindlin and Butler micromethod ('38) were made on the 7th, 8th, 11th, and 12th day of each period.

Urinary analyses for reduced ascorbic acid by the method of Evelyn, Malloy and Rosen ('38) were made for all subjects on

24-hour samples collected after the first voiding in the morning for the 42 days of the experiment. In three of the 4 experiments the total ascorbic acid of the urine was also determined by the 2,4-dinitrophenylhydrazine method of Roe and Kuether ('43).

TABLE 1

The experimental design, showing plan of supplementing the basal diet with ascorbic acid [1]

EXPERIMENTAL PERIODS	ASCORBIC ACID SUPPLEMENTS					
	Sequence [2]					
	1	2	3	4	5	6
	mg/day			*mg/day*		
Pre-experimental period	25	25	25	25	25	25
	Square I			Square II		
Period 1	50	75	100	50	75	100
Period 2	75	100	50	100	50	75
Period 3	100	50	75	75	100	50

[1] We are indebted to the Institute of Statistics of the University of North Carolina for the design used and to Dr. H. L. Lucas of the Institute for the statistical analyses and many helpful suggestions.

[2] Six subjects, one on each sequence, were studied during each experiment. The 4 experiments were carried out in the summers of 1945 and 1946 and the winters of 1946 and 1947.

RESULTS

Adjustment to changing intake

As a result of the findings of the earlier report, no adjustment to intake was expected before the last 6 days of the experimental period. To test the possibility that less than 12 days might be required, the average blood plasma ascorbic acid levels on the 7th and 8th days were compared with those of the 11th and 12th days. Since data on the urinary output were continuous, the average values for the first and for the last 6 days were compared, as well as the averages for the 7th and 8th days and for the 11th and 12th days, which corresponded to the periods of the blood plasma analyses. Similar data on the urinary excretion of total ascorbic acid are given

for the last three experimental groups for two winters and one summer. The data are shown in table 2. Total ascorbic acid values were greater than reduced ascorbic acid values. As excretion increased at the higher levels of intake the differences between total and reduced ascorbic acid tended to increase.

TABLE 2

Summary of mean ascorbic acid values found at different times within the experimental periods

TIME	ASCORBIC ACID SUPPLEMENTS [1]	MEAN URINARY ASCORBIC ACID		MEAN BLOOD PLASMA ASCORBIC ACID [2]
		Reduced [2]	Total [3]	
	mg/day	*mg/day*	*mg/day*	*mg/100 ml*
First 6 days	50	14.2	17.3	...
	75	17.8	21.6	...
	100	24.6	31.7	...
Standard error of a mean [4] — %		± 8.30	± 11.00	
7th and 8th days	50	11.1	14.6	0.75
	75	20.6	25.8	0.88
	100	37.7	47.4	1.03
Standard error of a mean — %		± 7.86	± 7.89	± 2.33
11th and 12th days	50	10.8	13.7	0.78
	75	24.8	30.5	0.96
	100	43.6	51.4	1.05
Standard error of a mean — %		± 6.50	± 8.69	± 2.63
Last 6 days	50	10.8	14.0	...
	75	21.8	26.2	...
	100	40.8	49.2	...
Standard error of a mean — %		± 5.78	± 6.28	

[1] The diet contained 6.9 mg reduced ascorbic acid and 11.5 mg total ascorbic acid.

[2] Average of 24 subjects, experiments 1–4.

[3] Average of 18 subjects, experiments 2–4.

[4] For standard error of any given mean multiply by the error figure for the group and divide by 100. Example: (24.6) (± 8.30)/100 = ± 2.04.

The data make it evident that adjustments to the varying increases and decreases of ascorbic acid differed and that previous intake had a marked effect on current blood values and urinary excretions of ascorbic acid, which effect was not eradicated in the 12 days of the experimental period. The preliminary 6 days on a 25-mg intake, used to train subjects

to routine and to minimize the effect of varying normal intakes, while not a part of the experimental design, introduced an increment of 75 mg for the subjects beginning the experiment at the 100-mg level. This was without a parallel decrease. At the 50- and 75-mg intake level, each period was preceded by a higher and a lower intake which tended to offset each other, while the 100-mg intake always followed lower intakes. The

TABLE 3

Mean daily urinary outputs and mean blood plasma levels of ascorbic acid during the 11th and 12th days as related to current and previous ascorbic acid intakes

ASCORBIC ACID SUPPLEMENTS		MEAN URINARY ASCORBIC ACID		MEAN BLOOD PLASMA ASCORBIC ACID [1]
Previous	Current	Reduced [1]	Total [2]	
mg/day	*mg/day*	*mg/day*	*mg/day*	*mg/100 ml*
	50	9.1	9.5	0.54
25	75	17.9	20.8	0.81
	100	27.3	36.0	0.99
	50	...	...	...
50	75	23.8	29.8	0.97
	100	52.1	58.8	1.05
	50	11.5	13.9	0.90
75	75	...	...	...
	100	51.4	59.4	1.10
	50	11.9	17.8	0.90
100	75	32.6	40.9	1.08
	100	...	...	...

[1] Eight subjects on each study.
[2] Six subjects on each study.

effect of this carry-over on current values, based on analyses of blood plasma and urine on the 11th and 12th days, is presented in table 3.

Calculated "equilibrium" values

Fourteen days were adequate time for adjustment to the 25-mg increases of ascorbic acid previously reported by this laboratory, but it is evident from the data in table 3 that 12 days were not enough time to attain "equilibrium" on the

variable sequence of intakes used. Statistical values for "equilibrium" were determined from the summarized data of table 3 to eliminate this carry-over effect of previous intake, and checked by regression methods. This "equilibrium" may be defined as the value obtained if the subject is kept at a given intake indefinitely. These data are presented in table 4. The values for the 50- and 75-mg intakes have a validity not shared by the values calculated for the 100-mg intake, since they were both preceded by lower and higher intakes. Also the

TABLE 4

Estimated mean "equilibrium" values[1] and the approximate 95% confidence intervals for the estimates

ASCORBIC ACID SUPPLEMENTS	URINARY REDUCED ASCORBIC ACID		URINARY TOTAL ASCORBIC ACID		BLOOD PLASMA ASCORBIC ACID	
	Mean "equilibrium"	95% confidence interval	Mean "equilibrium"	95% confidence interval	Mean "equilibrium"	95% confidence interval
mg/day	*mg/day*		*mg/day*		*mg/100 ml*	
50	10.7	± 1.8	11.2	± 2.3	0.79	± 0.10
75	28.5	± 4.0	37.0	± 5.2	1.04	± 0.07
100	52.0	± 9.6	60.0	± 12.6	1.16	± 0.09

[1] Value obtained should subject be maintained on given intake indefinitely.

data for blood and urine at the 100-mg level obtained by extrapolation, however valid statistically, have a different connotation physiologically since blood plasma values of ascorbic acid have an upper limit, while urinary excretions can increase indefinitely with intake.

A comparison of the summarized statistical data (table 4) with those obtained by direct measurement (table 2) on the 11th and 12th days indicates the trend of the findings throughout the total experiment. At the 50-mg level, intake was on the average inadequate and urinary excretions showed a lowering trend. At the two higher levels the trend with time was toward increasing values and the "equilibrium" means obtained by regression, interpolation and extrapolation methods were higher than the experimental values.

Urinary excretion

While adjustment to intake for the variable sequence of this experimental plan is incomplete and reflects previous intake, values based on total intake (amount given plus that in diet, 6.9 mg for reduced ascorbic acid, 11.5 mg for total) and urinary excretion should give an indication of the ascorbic acid "utilized" or retained by these subjects. Data for the experimental means and calculated "equilibrium" means should give a measure of the upper and lower limits of this "utilization." These data are presented in table 5, calculated to milligrams

TABLE 5

Average retention,[1] milligrams per kilogram of body weight, based on experimental values of the 11th and 12th days and calculated "equilibrium" values

ASCORBIC ACID SUPPLEMENTS	RETENTION OF REDUCED ASCORBIC ACID [2]		RETENTION OF TOTAL ASCORBIC ACID [3]	
	Experimental	"Equilibrium"	Experimental	"Equilibrium"
mg/day	*mg/kg*	*mg/kg*	*mg/kg*	*mg/kg*
50	0.82	0.82	0.82	0.86
75	1.01	0.94	0.96	0.86
100	1.12	0.97	1.03	0.88

[1] Retention = $\frac{\text{total ascorbic acid intake (food and supplements)} - \text{excreted ascorbic acid}}{56.8 \text{ (ave. weight)}}$.

[2] Experiments 1–4.

[3] Experiments 2–4.

per kilogram of body weight. These findings indicate that for the subjects of these experiments 75 mg were adequate for maintenance; that an increase to 100 mg was accompanied by an increase in urinary excretion; and that the retention was close to 1 mg per kilogram of body weight, whether measured by reduced or total ascorbic acid.

Effect of season

Foulger ('42) has suggested that, due to increased perspiration at elevated temperatures, loss of ascorbic acid in the summer is increased. Kirch, Cornbleet and Bergeim ('43),

who have induced sweating and analyzed sweat for ascorbic acid, have refuted this. The response criteria of the present studies — blood plasma levels and the urinary excretion of ascorbic acid — were examined for the possible effect of season. The results uniformly indicated that season had no effect on mean urinary output, either total or reduced, or on mean blood level of ascorbic acid.

The values given as means at all experimental intakes are:

	SUMMER (78°F.)	WINTER (58°F.)
Urinary ascorbic acid		
Reduced (mg)	25.6	24.4
Total (mg)	26.7	31.4
Blood plasma ascorbic acid (mg/100 ml)	0.97	0.89

SUMMARY

Twenty-four college women served as subjects in a study of the relation and adjustment of blood plasma level and urinary excretion of ascorbic acid to intake. The data were collected in 4 experimental units of 6 subjects each. Two units were studied in the summer and two in the winter.

The experimental plan provided for intakes of 50, 75, and 100 mg of ascorbic acid, augmented by that of a low ascorbic acid diet, in increasing and decreasing order in sequences which differed for each of the 6 subjects of a unit. The units were replicates. The adjustment period at each intake was 12 days. Blood plasma ascorbic acid analyses were made at stated intervals and urinary excretion analyses were made daily on 24-hour collections.

Both blood plasma and urinary excretion values of ascorbic acid, as criteria, indicated a carry-over effect of the previous intake which was still evident at the end of 12 days.

"Equilibrium" values were statistically estimated from the data and, based on the trends shown, indicate expected values at "equilibrium" had it been reached.

Retention calculated both from experimental data and estimated "equilibrium" values shows for these subjects a "utilization" close to 1 mg per kilogram of body weight.

No difference in requirement due to season is shown by the response criteria of this series of experiments.

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AVAILABILITY OF PANTHENOL AND CALCIUM PANTOTHENATE IN RELATION TO FOOD INTAKE[1]

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SIX FIGURES

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Loading tests with pantothenic acid have been carried out by various workers with human subjects (cf. Burlet, '44; Schmidt, '45; Sarett, '45; Rubin et al., '48) as well as with animals (Silber, '45; Olcese and Pearson, '48). Only a portion of the ingested doses has been found to be excreted as unchanged pantothenate; e.g., urinary excretion in the human accounted for 15–20% of oral doses of Ca pantothenate ranging from 100 to 250 mg (Rubin et al., '48). Similar percentage excretions were reported by Schmidt ('45) after intravenous injection of 25-mg doses of Ca pantothenate or panthenol. Silber ('45), who studied pantothenic acid excretion in dogs, and Olcese and Pearson ('48), working with sheep, found that some of the ingested Ca pantothenate was excreted unchanged in the feces. Silber ('45) demonstrated that the fecal loss was proportional to the amount of food taken with the dose. None of these studies, however, accounted for an excretion (urine and feces) of more than 50% of the ingested Ca pantothenate. In the present study, the rat was used as the test animal to compare excretion of pantothenic acid after the ingestion of loading doses of panthenol or Ca pantothenate under various feeding conditions. Panthenol,

[1] Presented before the American Institute of Nutrition, Detroit, April 21, 1949 (Fed. Proc., *8:* 393).

the alcohol analogue of pantothenic acid, although inactive for the growth of most microorganisms except *Acetobacter suboxydans* (Walter, '46; De Ritter and Rubin, '49), can replace pantothenate in the nutrition of the rat (Pfaltz, '43; Drekter et al., '48) and chick (Hegsted, '48; Milligan and Briggs, '49). Moreover, this compound is of considerable interest because of its greater availability to humans at high doses (Burlet, '44; Rubin et al., '48) and its greater stability *in vitro* as compared to Ca pantothenate, particularly in the pH range of 3 to 5 (Rubin, '48).

The fact that panthenol is as effective as the salts of pantothenic acid in curing pantothenic acid deficiency in the rat (Drekter et al., '48) provides a basis for interpreting excretion data in terms of availability.

EXPERIMENTAL

Adult, male albino rats of the Sprague-Dawley strain, weighing between 375 and 475 gm, were used for these studies. The animals were housed and the test was handled according to the procedure described by Drekter et al. ('48). The microbiological assay method for urinary and fecal pantothenic acid was that of Skeggs and Wright ('44), for panthenol that of De Ritter and Rubin ('49). Panthenol and Ca pantothenate were compared at all levels on an equivalent basis, all doses being administered by stomach tube in 1 ml of distilled water. In calculating extra urinary excretions, a "statistical" basal value (Drekter et al., '48) was subtracted from the observed excretions. Since these studies extended over a period of months, basal values were determined again at the end of that time and were found to check with the "statistical" value. Experiments were run at three dosage levels: 10, 5 and approximately 2 mg/kg; for each dosage level, 10 to 20 rats were used. The results were evaluated according to the formula for the standard error of the difference between two means.[2]

$$^{2}\sigma_D = \frac{m_1 - m_2}{\sqrt{\frac{d^2_1}{N_1} \frac{d^2_2}{N_2}}}.$$

Ad libitum feeding

Rats were allowed to eat ad libitum up to the time of ingestion of the dose and throughout the two 24-hour collection periods. On the average, 173, 85 and 7% more pantothenic acid was excreted in the urine in the 24 hours after the ingestion of panthenol at the 10, 5 and 1.25 mg/kg levels, respectively, than after ingestion of equivalent doses of Ca pantothenate (fig. 1). Only the differences at the 10 and 5 mg/kg levels are statistically significant ($P < 0.05$). Approximately 60% of the dose of panthenol was excreted as pantothenate in the urine in the first 24 hours at all three levels, whereas only 23 and 33% of the doses of Ca pantothenate were excreted in a similar interval at the 10 and 5 mg levels, respectively. At the lowest level (1.25 mg/kg), the percentage excretion approximated that which occurred after giving panthenol. In the second 24-hour interval approximately 5% of the dose of both panthenol and Ca pantothenate was found in the urine at the two highest levels; hence, second-day values exert little effect in any of these experiments.

Controlled feeding experiments

In order to produce conditions under which the effect of food on the relative availability of panthenol and Ca pantothenate could be studied, food was withheld from rats for 24 hours prior to dosage. At the end of this starvation period, the test group were given 6 gm of stock diet (approximately two-fifths of the daily food intake); the rats consumed the food within 45 minutes. The doses were administered to these and to the unfed control rats (postabsorptive state) immediately thereafter. The collection of urine and feces was begun, and after 6 hours all rats were given stock diet. Basal urinary and fecal excretions were determined under similar conditions. The basal urinary excretion was found to be the same regardless of whether or not the rats were fed at the start of the collection period. This basal value

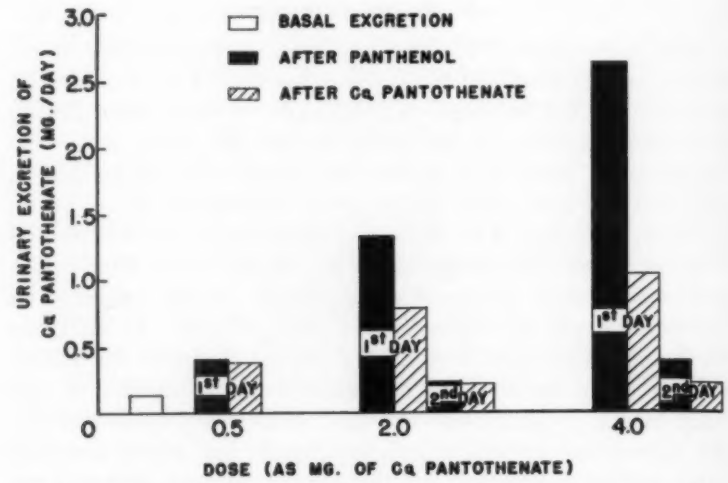

Fig. 1 Urinary excretion of Ca pantothenate after various equivalent doses of panthenol and Ca pantothenate to rats fed ad libitum.

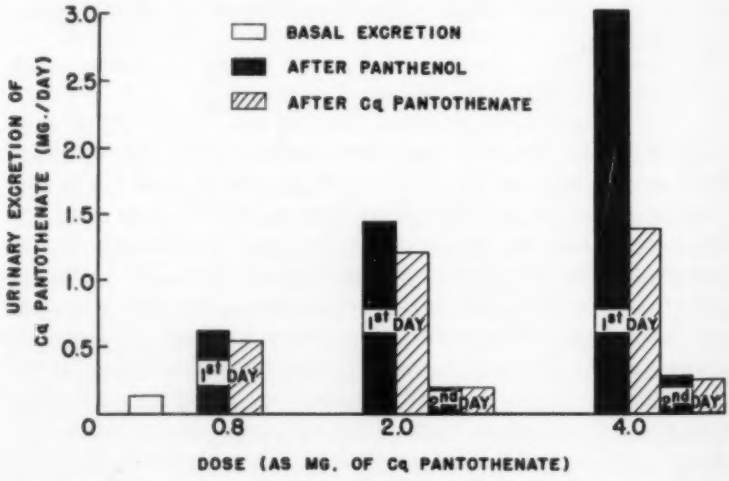

Fig. 2 Urinary excretion of Ca pantothenate after various equivalent doses of panthenol and Ca pantothenate — postabsorptive.

(0.14 mg/day) was similar to that found in the ad libitum experiments. No correlation was noted between the amount of pantothenic acid excreted and the volume of urine. The amount of pantothenic acid in the feces was found to be directly proportional to the weight of the feces. Therefore, to calculate extra fecal excretions, basal values were subtracted in proportion to the amount of feces.

After postabsorptive dosage with panthenol, 131, 24 and 15% more pantothenic acid was excreted in the urine at the 10, 5 and 2 mg/kg levels, respectively, than after dosage with Ca pantothenate (fig. 2). Only the difference at the 10 mg/kg level was significant. Under these feeding conditions, 74, 67 and 59% of the doses of panthenol and 32, 54 and 51% of the doses of Ca pantothenate were excreted at the various levels. When the doses were administered to rats in the postprandial state, the excretion of pantothenic acid after giving panthenol exceeded the excretion after administering Ca pantothenate by 92, 45 and 2% (fig. 3). The differences at the 10 and 5 mg/kg levels were significant. Sixty-seven, 61 and 49% of the doses of panthenol were recovered in the urine as pantothenic acid, as compared to 35, 42 and 48% of the doses of Ca pantothenate.

The presence of food at the time of dosage had no significant effect on the urinary excretion after dosage with either Ca pantothenate (fig. 4) or panthenol (fig. 5). However, fecal excretion of Ca pantothenate was affected by food intake (fig. 6). A significantly greater fecal excretion of pantothenic acid was found when a dose of 10 mg/kg of Ca pantothenate was given simultaneously with food than when food was absent (30 vs. 16%). At the 5 mg/kg level, the presence of food increased the fecal excretion of pantothenic acid slightly, but the effect was not statistically significant. At the lowest level (2 mg/kg), no extra fecal excretion could be demonstrated in either case. At the two higher levels some extra pantothenic acid was excreted in the feces (16 and 7%) even when food was absent at the time of ingestion of the dose. No increase in the fecal excretion of pantothenic

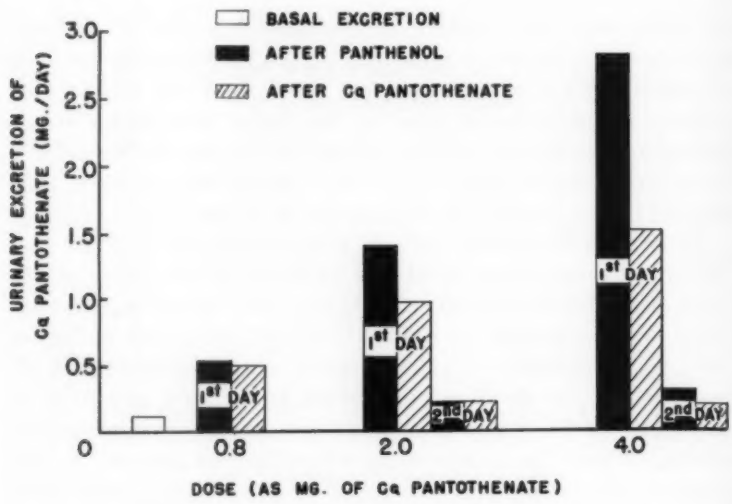

Fig. 3 Urinary excretion of Ca pantothenate after various equivalent doses of panthenol and Ca pantothenate — postprandial.

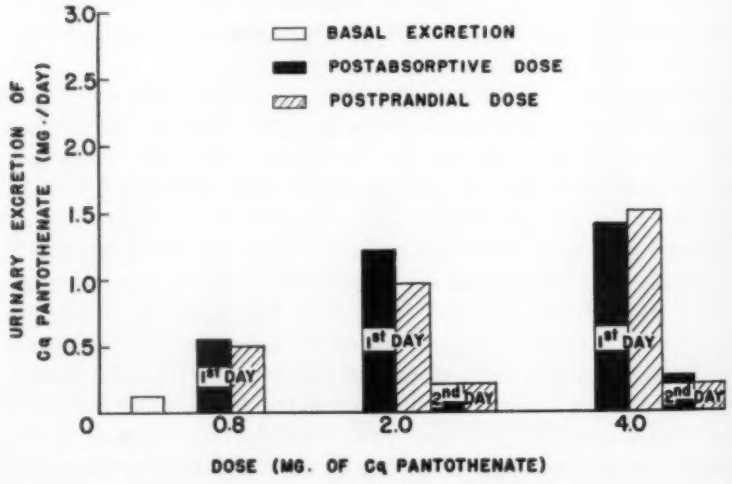

Fig. 4 Urinary excretion of Ca pantothenate after various doses of Ca pantothenate — postabsorptive and postprandial.

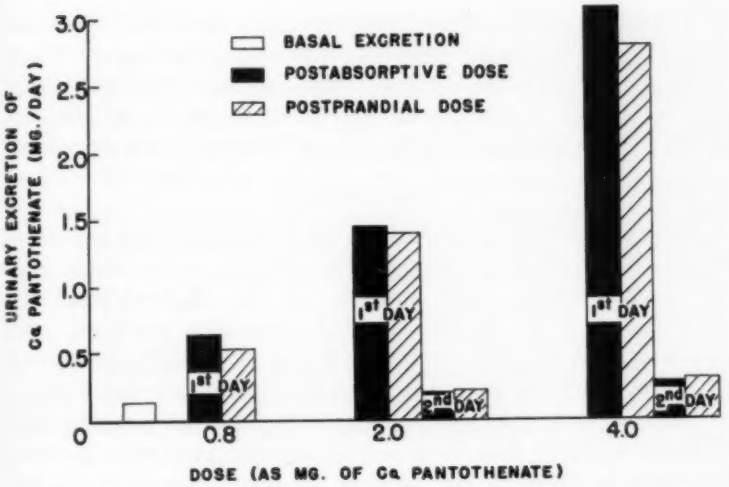

Fig. 5 Urinary excretion of Ca pantothenate after various doses of panthenol — postabsorptive and postprandial.

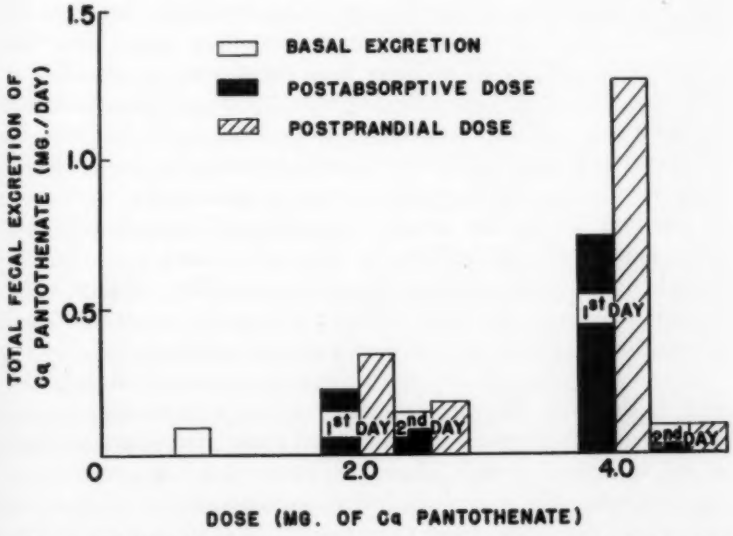

Fig. 6 Effect of food upon fecal excretion after various doses of Ca pantothenate — postabsorptive and postprandial.

acid was observed when panthenol was fed, an indication that no conversion of panthenol to pantothenic acid takes place in the gastrointestinal tract, nor was any panthenol per se found in the feces under any feeding conditions. Second-day excretion values did not change these trends.

DISCUSSION

The excretion of pantothenic acid by the rat after ingestion of equivalent doses of panthenol and Ca pantothenate follows the same pattern as in humans. At the highest level fed (10 mg/kg), which is considerably above the top level used in our human availability tests (Rubin et al., '48), panthenol is more available than Ca pantothenate in all three types of feeding tests. At the intermediate level of 5 mg/kg this difference exists but to a lesser degree, particularly when the dose is given in the absence of food. Similarly, in the human availability tests at approximately the same milligram per kilogram dosage, panthenol was much more available than Ca pantothenate when given postprandially, but equally available when given in the postabsorptive state. At the lowest level fed (1.25 or 2 mg/kg) there was no significant difference in excretion after dosage with panthenol or Ca pantothenate. This parallels the findings with humans at comparable dosage levels as well as those noted in unpublished experiments in which doses as low as 0.4 mg/kg were fed. At the high levels, the greater excretion of pantothenic acid after panthenol administration than after giving Ca pantothenate may be explained by the greater stability of panthenol in acid solutions (Rubin, '48), resulting in better survival of panthenol during the gastrointestinal journey.

Silber ('45) has shown with dogs that when food is given with a dose of Ca pantothenate, urinary excretion is decreased and fecal excretion increased proportionately as compared to values found when the same dose is given postabsorptively. He also found that increasing the amount of food given increased fecal excretion of pantothenic acid. The proportion of fecal to urinary excretion increased from 1.3,

when no food was given, to 20 with the largest amount of food. Olcese and Pearson ('48) found more pantothenic acid in the feces than in the urine when sheep were given 2,000 mg of Ca pantothenate. In the present experiments with rats there was no decrease in the urinary excretion of pantothenic acid in the presence of food, but increased fecal excretion did occur. The amount of pantothenic acid excreted in the feces, even in the presence of food, was never larger than that excreted in the urine. It appears that the rat can absorb considerably more of this vitamin than either the dog or the sheep. Not only is less excreted in the rat feces, but also a larger proportion of a test dose appears in the urine (from 23–58% in the rat as compared to 2.2–16% in the dog and approximately 4% in the sheep), irrespective of whether food is given with the dose or not.

CONCLUSIONS

1. Under all feeding conditions panthenol is more available than Ca pantothenate, as judged by urinary excretion, when fed to rats at a level of 10 mg/kg.

2. At an intermediate level (5 mg/kg), panthenol has significantly superior availability when administered to rats fed ad libitum or in the postprandial state, the differences being 85 and 45%, respectively. When the doses were administered in the postabsorptive state, the difference was 24%, which is not statistically significant.

3. At the lowest level (2 or 1.25 mg/kg), no significant differences in the urinary excretion of pantothenic acid were found between panthenol and Ca pantothenate, regardless of the immediately previous nutritional status of the animals.

4. Recoveries of pantothenic acid in the urine in the 24 hours after dosage range from 49 to 74% of the dose of panthenol and 23 to 58% of the dose of Ca pantothenate.

5. The presence of food at the time of ingestion of either panthenol or Ca pantothenate does not materially affect the urinary excretion of pantothenic acid. However, in the case of Ca pantothenate at the highest level of dosage (10 mg/kg),

the presence of food causes a significantly increased excretion of fecal pantothenic acid. At the intermediate level (5 mg/kg) there is also an increase in fecal excretion when food is present, but this is not statistically significant. At both these levels some of the ingested Ca pantothenate is excreted in the feces even when food is absent. At the lowest level (2 mg/kg) no increase in fecal pantothenic acid was observed.

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ABSORPTION OF AQUEOUS DISPERSIONS OF VITAMIN A ALCOHOL AND VITAMIN A ESTER IN NORMAL CHILDREN [1]

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From a comparative study of the absorption of oily preparations of vitamin A alcohol and vitamin A ester, McCoord et al. ('48) concluded that there is little difference between the absorption of the two forms of vitamin A in oil. In a similar study using oily preparations of vitamin A ester and alcohol in normal newborn infants, Sobel et al. ('49) also demonstrated that there is no significant difference in absorption of either form of vitamin A when administered in oil.

It has been conclusively demonstrated that when given orally vitamin A alcohol in aqueous dispersion is more readily absorbed than is vitamin A ester in oil (Sobel et al., '49; Kramer et al., '47; Lewis et al., '47). The purpose of the present study, therefore, was to determine whether it is the alcohol form of the vitamin as opposed to the ester form which is responsible, or whether it is the physical state of the vitamin, i.e., the fact that it is in aqueous dispersion rather than in oil, which is the more important factor. Vitamin A ester in the form of palmitate was therefore prepared in an aqueous dispersion and administered to 15 children. Vitamin A alcohol similarly dispersed was given to the same children, as described below. The vitamin concentrations attained in the blood serum at varying intervals after each preparation were measured.

[1] This study was aided by a grant from Hoffmann-La Roche, Inc., Nutley, N. J.

MATERIAL AND METHODS

Vitamin A palmitate and vitamin A alcohol were specially prepared for the study.[2] Each preparation contained 25,000 units/ml and was dispersed with 30% Tween 80.[3]

The children were apparently normal individuals convalescing from minor surgical procedures. Each received two test doses of 6,000 units of vitamin A per pound of body weight. Half of the subjects were given the palmitate first and the other half were given the alcohol form first. A rest period of at least 48 hours was allowed between the times the test doses were given. No difference in the second test was detected which could be attributed to the effect of the preceding test dose.

Serum vitamin A concentrations were determined by a minor modification of the Carr and Price method as described by Sobel et al. ('49) and Kagan et al. ('50).

RESULTS

Table 1 gives the serum vitamin A values obtained in the fasting state and at three, 6 and 24 hours after the test dose.

Table 2 shows the differences in the levels obtained with the palmitate form and with the alcohol. The differences between the serum vitamin A concentrations obtained following vitamin A palmitate and those following vitamin A alcohol are shown for each interval of time. (Values following vitamin A alcohol were substracted from those following vitamin A palmitate.) Ten of the 15 subjects had less serum vitamin A at the three-hour interval following the palmitate form than at the same interval following the alcohol form. The mean difference at three hours was — 401 U.S.P. units/100 ml. Twelve of the 15 subjects had higher serum vitamin A values at the 6-hour interval following the palmitate form

[2] Through the cooperation of Dr. Elmer L. Sevringhaus and Dr. M. J. Schiffrin of Hoffmann-La Roche, Inc.

[3] Polyoxyalkalene derivative of sorbitan monolaurate.

TABLE 1

Serum vitamin A concentrations following administration of 6,000 U.S.P. units per pound body weight of vitamin A alcohol and vitamin A palmitate in aqueous dispersion

CASE	FORM OF VITAMIN A	SERUM VITAMIN A CONCENTRATIONS			
		Fasting	3 Hours	6 Hours	24 Hours
		U.S.P. units/100 ml			
1 D.Q.	Alcohol	120	1,002	693	100
	Palmitate	93	2,398	882	210
2 S.M.	Alcohol	107	2,444	706	200
	Palmitate	93	3,956	889	186
3 T.L.	Alcohol	83	3,626	539	153
	Palmitate	110	2,198	999	200
4 K.C.	Alcohol	140	5,145	859	250
	Palmitate	180	4,496	1,878	440
5 B.L.	Alcohol	123	2,697	360	250
	Palmitate	170	4,555	1,382	170
6 J.R.	Alcohol	100	1,698	363	100
	Palmitate	90	1,698	626	150
7 G.H.	Alcohol	160	5,195	783	290
	Palmitate	210	1,898	1,156	240
8 N.M.	Alcohol	180	2,448	676	140
	Palmitate	150	1,975	842	180
9 J.R.	Palmitate	140	2,974	1,069	150
	Alcohol	133	3,440	1,069	223
10 R.B.	Palmitate	100	2,817	3,463	470
	Alcohol	60	4,362	1,618	316
11 G.R.	Palmitate	70	4,356	539	200
	Alcohol	160	4,835	559	250
12 C.S.	Palmitate	93	3,147	1,079	180
	Alcohol	110	1,758	713	153
13 L.S.	Palmitate	100	1,748	1,545	210
	Alcohol	143	2,316	689	140
14 C.R.	Palmitate	140	1,119	513	220
	Alcohol	150	2,085	509	210
15 C.J.	Palmitate	140	1,249	599	140
	Alcohol	130	3,530	1,648	140
Mean	Alcohol	126	3,105	785	194
	Palmitate	125	2,706	1,258	223
Standard deviation	Alcohol	31	1,310	388	68
	Palmitate	40	1,174	742	98
Standard error	Alcohol	8	338	100	18
	Palmitate	10	303	192	25

than at the same interval following the alcohol form. The mean difference at this interval was + 378 U.S.P. units/100 ml. While this suggests that the alcohol form may be absorbed somewhat faster or may be stored or utilized somewhat faster, or both, the differences in the results are such as to lack statistical significance. At 24 hours after the test dose the results with the two preparations were essentially the same.

TABLE 2

Vitamin A palmitate in aqueous dispersion compared to vitamin A alcohol in aqueous dispersion. Serum concentration differences (palmitate values minus alcohol values) in individual subjects following a dose of 6,000 U.S.P. units per pound body weight

	FASTING	3 HOURS	6 HOURS	24 HOURS
Number of cases	15	15	15	15
Mean difference [1]	— 1.3	— 401	+ 378	+ 29
Standard error	10	378	166	10

[1] According to the "t" test, the probabilities of the differences between the levels obtained with the two vitamin A preparations being due to sampling variation alone are 0.2 at 0 hour, 0.3 at 3 hours, 0.04 at 6 hours and 0.2 at 24 hours. According to convention a probability of less than 0.05 is assumed to be statistically significant (Snedecor, '46).

CONCLUSION

There is no significant difference in the serum vitamin A concentrations following single equal doses of either vitamin A alcohol in aqueous dispersion or vitamin A palmitate in aqueous dispersion. It is therefore the fact that the vitamin is prepared in an aqueous dispersion rather than in oil, and not the alcohol or ester form, which is responsible for increased absorption.

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STUDIES ON THE NUTRITIONAL ADEQUACY OF ARMY COMBAT RATIONS [1]

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During the war extensive studies were made on the composition of army rations, and these analyses indicated that the better known nutrients were present in quantities sufficient to supply daily dietary requirements (QMC studies, '44). Although the requirement for certain nutrients for the rat may be higher than that for man, animal feeding experiments using these complex army mixtures may yield information which would be of value in improving the formulation of rations for human consumption.

Most animal studies have involved purified rations in which one component is altered at a time. Relatively little attention has been given to the requirements for certain nutrients in natural rations or to the possible changes in the nutritional value of mixed diets when the latter are subjected to heat treatment. Army rations not only contain a variety of products but are processed rather thoroughly, so that they will withstand storage for long periods of time.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned number 270 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of the Army.

Previous work (Sporn and Elvehjem, '48; Sporn et al., '48) has shown that young rats and monkeys failed to grow normally, and the rats failed to reproduce successfully, when fed army rations such as K, 10-in-1 and C as the sole article of diet. These results were not too surprising, since several workers have found that natural foods combined according to the pattern of the American diet do not produce maximum growth and normal reproduction in the rat (Winters, '47).

Sporn and Elvehjem ('48) reported that good growth could be obtained when rats were fed army rations supplemented with a mixture of the known B vitamins and 5% casein. In studies with monkeys fed the K ration it was observed that supplements of pyridoxine and folic acid produced significant growth responses and that pyridoxine gave the largest response (Sporn et al., '48). This work has been continued in order to determine which of the B vitamins and which amino acids will replace the B vitamin mixture and casein supplements.

EXPERIMENTAL

Male, weanling rats (Sprague-Dawley) weighing between 40 and 45 gm were used in all growth experiments. Each experimental group consisted of 5 rats. The components of the rations were ground together and mixed in a Hobart mixer. The rations were stored at 0°C. and fed daily to the rats. The food cups were cleaned each day to avoid decomposition of the rations. When the rations were supplemented with the B complex vitamins the following levels in milligrams per 100 gm of ration were used: thiamine 0.3, riboflavin 0.3, niacin 2, pyridoxine 0.2, pantothenic acid 2, folic acid 0.025, biotin 0.01, inositol 10, choline 100 and *p*-aminobenzoic acid 25.

To determine the effect of omitting one of the vitamins, the K ration supplemented with 5% casein was used as a basal ration to which were added all the B vitamins except one. When pyridoxine, pantothenic acid, or biotin was omitted

from the supplement, maximum growth was not obtained (table 1). These vitamins were then added, singly and in combination, to the K ration with and without a casein supplement.

Pyridoxine accounted for the growth response given by the B vitamin mixture when added to the K ration and for most of the activity of the added B complex vitamins in the ration supplemented with casein. Pantothenic acid had no

TABLE 1

Effect of B vitamins and casein on the growth of rats fed the K ration

RATION AND SUPPLEMENTS FED	GAIN/WEEK 4-WEEK PERIOD
	gm.
K ration	15
K ration + B vitamin mixture	20
K ration + 5% casein	18
K ration + casein + B vitamins	32 ± 3
K ration + casein + B vitamins except B_6	21
K ration + casein + B vitamins except P.A.	25
K ration + casein + B vitamins except biotin	22
K ration + B_6	20
K ration + P.A.	14
K ration + B_6 + P.A.	20
K ration + B_6 + P.A. + biotin	19
K ration + 5% casein + B_6	29
K ration + 5% casein + B_6 + P.A.	35
K ration + 5% casein + B_6 + P.A. + biotin	33
K ration + 5% casein + P.A.	22

effect when added to the K ration alone, but always produced a slight growth response when the ration contained casein and pyridoxine. Biotin had no effect when added to the ration alone or to the ration supplemented with casein. The combination of pyridoxine and pantothenic acid gave a growth response equivalent to that produced by the B vitamin mixture when added to the casein-supplemented ration.

Since vitamin-amino acid relationships are known to exist, the B vitamin mixture was incorporated into the ration and

single amino acid additions were made. Obviously, the addition of all the amino acids except one to each group would involve excessive cost. Sporn and Elvehjem ('48) have shown that the addition of methionine to the K ration gave a small growth response in rats, so the K ration containing the B vitamin mixture and methionine was used as a basal ration to which were added other amino acids. Glutamic acid was included in one group to determine if the effect might be due merely to additional nitrogen in the ration. The addition of

TABLE 2

Effect of amino acid supplements added to K ration fed to rats

RATION AND SUPPLEMENTS FED	GAIN/WEEK 4-WEEK PERIOD
	gm
K ration + B vitamins + 0.2% DL-methionine	20
K ration + B vitamins + 0.2% DL-methionine + 0.1% DL-tryptophan	23
K ration + B vitamins + 0.2% DL-methionine + 0.5% L-leucine	27
K ration + B vitamins + 0.2% DL-methionine + 0.6% DL-threonine	24
K ration + B vitamins + 0.2% DL-methionine + 0.6% L-glutamic acid	23
K ration + B vitamins + 0.2% DL-methionine + 0.3% DL-phenylalanine	22
K ration + B vitamins + 0.2% DL-methionine + 0.6% L-lysine-HCl	21
K ration + B vitamins + 0.2% DL-methionine + 0.25% L-histidine-HCl	21
K ration + B vitamins + 0.2% DL-methionine + 0.1% DL-tryptophan, 0.5% L-leucine and 0.6% DL-threonine	26
K ration + B vitamins + 0.2% DL-methionine + 0.1% DL-tryptophan, 0.5% L-leucine, 0.6% DL-threonine and 6% L-glutamic acid	16

leucine appeared to produce the most pronounced effect, while tryptophan, threonine and glutamic acid produced only a slight increase in growth. The 4 essential amino acids giving the best response were then added together, with and without glutamic acid. No enhanced effect over that obtained with the ration containing methionine and leucine was observed. Glutamic acid caused a depression in growth in the presence of these additional amino acids (table 2). These results indicate that this approach is not suitable for determining which combination of amino acids can replace casein.

Fairly good growth was obtained when rats were fed the C_2 ration; in fact, almost as good as that obtained with the other rations when supplemented with both casein and the B vitamins. It is by far the best army ration tested in our laboratory. Single supplements of casein or the B vitamin mixture had little effect on the growth of rats; however, when the two were added together improved growth was obtained (table 3).

TABLE 3

Effect of supplements added to the C_2 ration fed to rats

RATION AND SUPPLEMENTS FED	GAIN/WEEK 4-WEEK PERIOD
	gm
C_2 ration	28
C_2 ration + 5% casein	29
C_2 ration + B vitamin mixture	30
C_2 ration + B vitamin mixture + 5% casein	37
C_2 ration + 5% casein + B vitamins except B_6	34
C_2 ration + 5% casein + B vitamins except P.A.	35
C_2 ration + 5% casein + B_6	32
C_2 ration + 5% casein + P.A.	31
C_2 ration + 5% casein + B_6 + P.A.	35
C_2 ration + methionine + B_6 + P.A.	34
C_2 ration + methionine + B_6	30
C_2 ration + methionine	28
C_2 ration + B_6	30
C_2 ration + P.A.	26
C_2 ration + B_6 + P.A.	28

When the C_2 ration supplemented with casein and all the B vitamins except pantothenic acid or pyridoxine was fed, slightly less growth was obtained. When these two vitamins were added with casein, they accounted for most of the activity observed with the B vitamin mixture. If pyridoxine or pantothenic was added, alone or together, to the unsupplemented C_2 ration, little effect on growth could be observed. Although methionine had no effect when added to the unsupplemented ration, it produced a marked growth response when added to the ration containing pyridoxine and panto-

thenic acid, and appeared to replace to a great extent the casein supplement.

Studies with the 10-in-1 ration have shown that pyridoxine will replace the B vitamin mixture in the casein-supplemented ration (table 4). Due to the unavailability of lists of all the components of the 10-in-1 ration, its study has been temporarily discontinued.

The protein, moisture, pyridoxine and pantothenic acid contents of the K and C_2 rations are shown in table 5. These suggest that the K ration is definitely low in protein for the growing rat. Microbiological assays indicate that there is a sufficient quantity of pyridoxine and pantothenic acid

TABLE 4

Effect of supplements on the growth of rats fed the 10-in-1 ration

RATION AND SUPPLEMENTS FED	GAIN/WEEK 4-WEEK PERIOD
	gm
10-in-1 ration	19
10-in-1 ration + B vitamin mixture	20
10-in-1 ration + B_6	19
10-in-1 ration + 5% casein	23
10-in-1 ration + 5% casein + B vitamin mixture	32
10-in-1 ration + 5% casein + B vitamin mixture except B_6	23
10-in-1 ration + 5% casein + B_6	31

in the K and C_2 rations, if these vitamins are completely available for the animal and if the ration is eaten at a normal rate of 10 gm per day.

The food consumption of rats fed the C_2 and K rations with and without supplements was measured, to determine the effect of these supplements (table 6). Rats fed the C_2 ration consumed a normal amount, 10 gm per day, while those on the K ration ate only about one-half as much. When vitamins were added to the K ration, the food intake increased 26%; however, there was no difference in the protein efficiency whether the vitamins were omitted or added. The addition of pyridoxine alone to the K ration also produced an increased food intake, with a corresponding increase in

growth to 20 gm per week. When casein was added alone as a supplement, there was no effect on the food intake, but when it was added to the ration supplemented with pyridoxine the food intake increased, with a resulting increase in weight gain. During the 5th week of another experiment the food intake of rats fed the casein-supplemented ration was 6.3 gm,

TABLE 5

Moisture, protein, pyridoxine and pantothenic acid content of the K and C_2 rations

RATION	MOISTURE	DETERMINED ON A DRY WEIGHT BASIS		
		Protein	Pyridoxine	Pantothenic acid
		%	*mg/100 gm*	*mg/100 gm*
C_2	55	19.0	0.262	1.07
K	25	12.5	0.145	1.27

TABLE 6

Food consumption of rats fed the K and C_2 rations

RATION	AV. GAIN, 4-WK. PERIOD	RATION EATEN PER DAY, DRY WEIGHT	PROTEIN PER DAY	GAIN PER GRAM PROTEIN EATEN
	gm	*gm*	*gm*	*gm*
K	15	6.1	0.76	2.82
K + B vitamins	19	7.7	0.96	2.83
K + B_5 + casein	26	8.6	1.55	2.4
C_2 ration	28	10.0	1.9	2.11

while those fed the ration supplemented with casein and pyridoxine ate 11.7 gm. The average gain per week of the two groups for the 5-week period was 19 and 30 gm, respectively.

To determine the quality of protein, a microbiological assay of the amino acids was made on the K and C_2 rations (table 7). According to the requirements given by Rose for the rat, most of the essential amino acids were low. Applying

these results, rats were fed the K ration supplemented with the 6 amino acids shown to be low. This amino acid mixture replaced casein in promoting growth when pyridoxine or pyridoxine and pantothenic acid were incorporated as vitamin

TABLE 7

Amino acid content of K and C_2 rations expressed as a percentage on a dry weight basis

AMINO ACID	K RATION	TENTATIVE REQ. FOR RATS (ROSE)	C_2 RATION
Phenylalanine	0.53	0.7	0.77
Tryptophan	0.08	0.2	0.14
Histidine	0.27	0.4	0.45
Lysine	0.64	1.0	1.09
Threonine	0.48	0.6	0.74
Methionine	0.41	0.6	0.59
Leucine	1.00	0.9	1.32
Valine	0.72	0.7	1.00
Isoleucine	0.52	0.5	0.79
Arginine	0.55	0.2	0.99

TABLE 8

A comparison of the effect of the casein and the amino acid mixture on the growth of rats fed the K ration

RATION AND SUPPLEMENTS FED	GAIN/WEEK 4-WEEK PERIOD
	gm
K ration + B_6 + casein	26
K ration + B_6 + P.A. + casein	28
K ration + B_6 + amino acid mixture [1]	29
K ration + B_6 + P.A. + amino acid mixture	30
K ration + B_6 + amino acid mixture less threonine	20
K ration + amino acid mixture	20
K ration + amino acid mixture + 0.5% L-leucine + B_6	29

[1] Phenylalanine 0.3%, tryptophan 0.2%, lysine 0.4%, threonine 0.15%, methionine 0.3,% histidine 0.2%.

supplements (table 8). The amino acid mixture without the vitamins had no greater effect than casein without the vitamins. When threonine was omitted from the amino acid mixture the effect of the added amino acids was completely

nullified. Whether the omission of one of the other 5 amino acids would prevent maximum growth remains to be determined. The addition of leucine to the amino acid mixture had no effect.

Although the assay showed that tryptophan is low in the C_2 ration, its addition to the ration with or without added vitamins had no effect on the growth of rats.

When the amino acid content of the protein in the K ration is compared with that in the C_2 ration, a similarity is evident (table 9). However, lower amounts of each amino acid are

TABLE 9

Amino acid content of K and C_2 rations expressed as percentage in the protein

AMINO ACID	K RATION	C_2 RATION	WHOLE EGG	CASEIN
Phenylalanine	4.2	4.1	6.3	5.2
Tryptophan	0.6	0.7	1.5	1.8
Lysine	5.1	5.7	7.2	6.9
Isoleucine	4.1	4.2	8.0	6.5
Methionine	3.3	3.1	4.1	3.5
Leucine	8.0	6.9	9.2	12.1
Valine	5.7	5.2	7.3	7.0
Histidine	2.2	2.3	2.1	2.5
Arginine	4.4	5.2	6.4	4.1
Threonine	3.8	3.9	4.9	3.9

supplied by the K ration, since it is lower in total protein. The level of most of the amino acids is lower in the protein of the army rations than in whole egg or casein. This is to be expected, since the army rations contain a variety of protein sources, including vegetable proteins.

DISCUSSION

In all the army rations tested, and especially the K and 10-in-1 rations, using the rat as the experimental animal, pyridoxine is the most important vitamin supplement, since it replaces to a very large extent the B vitamin mixture. It is not fully understood why there is a need for additional

vitamin B_6, since microbiological assays indicate that there should be sufficient vitamin B_6 in both the K and C_2 rations. This statement is based on the observations of Sarma et al. ('46), who showed that 100 μg of pyridoxine per 100 gm of ration would give excellent growth in rats fed a purified ration, and that 150 μg would produce maximum growth. These results are also based upon a normal intake of food.

That constituents of the diet may affect the vitamin B_6 requirement of the rat was shown by the addition of dextrin, in place of sucrose, to a vitamin B_6-deficient diet (Sarma, Snell and Elvehjem, '47). The rats on the dextrin diet grew as well as those fed the sucrose diet when the ration contained 50 μg of pyridoxine per 100 gm of ration. Sarma and coworkers presented convincing evidence that intestinal synthesis of vitamin B_6 occurred when dextrin was fed. One might expect such a favorable condition to exist also when these army rations are used, since they contain wheat biscuits which have been dextrinized to some extent.

However, the adverse effects of certain dietary constituents on the vitamin B_6 requirement of experimental animals have been observed. Kratzer and Williams ('48) showed that the addition of 30% linseed oil meal to the ration of chicks caused a 5-fold increase in the requirement for vitamin B_6. Sarma et al. ('47) found that oleic acid or a high level of tryptophan or methionine fed to rats exerted a growth retarding effect when added to rations containing suboptimum amounts of pyridoxine.

Cerecedo and Foy ('44) presented data indicating that rats on a vitamin B_6-deficient diet developed earlier deficiency symptoms and more severe lesions on a high protein diet. Similar work was done by Miller and Baumann ('45a), but they found little difference in the survival time of rats on a pyridoxine-deficient diet fed either 20 or 60% casein.

Sure and Romans ('48) showed that the level of B vitamins in a purified ration had a pronounced effect on food intake, food utilization, protein efficiency ratio and the biological value of the protein. When vitamin B_6-deficient rats

on a controlled food intake were given vitamin B_6, they gained twice as much as those not receiving the vitamin (Sure and Easterling, '49). The increased food intake in the present experiments when the B vitamin mixture or pyridoxine was added to the K ration is in agreement with the results of Sure and associates; however, there was little or no change in the protein efficiency ratio or in food utilization. This difference is no doubt due to the fact that Sure was working with deficient animals, using a low vitamin B_6 diet as a control, whereas the K ration probably contained sufficient vitamin B_6 to permit good food utilization. Thus the chief effect of adding B_6 to the K ration appears to be an increased food intake, with a corresponding increase in weight gain.

The values given for the vitamin B_6 in table 4 represent the total amount present in the ration, which includes the three active forms. Since Miller and Baumann ('45b) have reported that pyridoxal and pyridoxamine are less active than pyridoxine when added to a ration for mice and rats, the present assay does not measure the total active vitamin B_6 for the rat. These results were confirmed by Sarma and co-workers ('47). The actual amount of active vitamin B_6 is further reduced when it is considered that Rabinowitz and Snell ('48) found that vitamin B_6 existed in natural products chiefly in the less active forms.

The explanation for the increased requirement of vitamin B_6 in rats fed the K ration must await further experimentation. The role that pantothenic acid may play in rendering these rations more adequate is also not clear at present. Nelson and Evans ('49) have shown that methionine has a marked sparing action on the requirement of pantothenic acid by the rat. This may explain the reason for no additional growth when pantothenic acid was added to the K ration supplemented with pyridoxine and amino acids, including methionine. The effect of added pantothenic acid is small as compared with that of added pyridoxine, and may well be termed a stimulatory effect rather than a significant growth response.

Riesen et al. ('46) showed that the food intake of rats on an 18% casein ration was 30% higher than that on an 8% casein ration. This observation may explain to some extent the lower food intake of the K ration as compared to that of the ration with added protein or to that of the C_2 ration, which contains a higher level of protein.

The growth response observed when methionine was added to the K and C_2 rations may be attributed to one or a number of factors. Riesen and associates ('46) observed that rats on a low protein diet supplemented with methionine or cystine consumed slightly more food and gained almost twice as much as those on the unsupplemented ration. Brush and co-workers ('47) demonstrated that methionine is superior to all other indispensable amino acids in its ability to decrease the loss of tissue protein in rats fed low protein diets. They concluded that it is more important in the synthesis of indispensable metabolites or of functional proteins than in the maintenance of the structural tissues of the body. This tissue-sparing effect of methionine, however, could not be demonstrated in human subjects (Johnson et al., '47).

The present studies on army rations indicate the importance of expanding laboratory work on the requirements of experimental animals for the essential dietary components when mixed diets are used instead of purified rations.

SUMMARY

Army combat rations K, C_2 and 10-in-1 were fed ad libitum to young growing rats. The materials fed represent only a limited sampling, since the items included in the rations have been changed from time to time. The growth of young rats over short periods of time was the chief criterion used in these experiments for evaluating the rations.

The growth response previously observed with the addition of the B complex to the K ration can be duplicated to a large extent with B_6. Pyridoxine completely replaced the B complex as a supplement to the 10-in-1 ration.

Pantothenic acid had no effect on growth of rats on the 10-in-1 ration supplemented with casein and pyridoxine, but did exert a small but consistent response when added to K and C_2 rations containing the same supplements. The two vitamins appeared to replace the B complex supplement.

The addition of 6 essential amino acids produced a growth response equal to that obtained when casein was added to the K ration supplemented with pyridoxine and pantothenic acid. In the C_2 ration, methionine appeared to replace the casein supplement.

The requirements for certain nutrients of experimental animals such as the growing rat are higher than those of man; therefore these results do not necessarily reflect the nutritional adequacy of these rations for human subjects.

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THE RELATION OF THE AMINO ACID-SUGAR REACTION TO THE NUTRITIVE VALUE OF PROTEIN HYDROLYSATES

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ONE FIGURE

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The deleterious effect of heat treatment on the biological value of proteins (heat injury) is well known. It has been the subject of continuous study since the report by McCollum and Davis ('17) on the decreased nutritive efficiency of heated milk. The effect of dry heat, autoclaving and cooking on the nutritive value of a variety of proteins and protein sources has been reported from many laboratories (Davis et al., '49).

In recent years the reaction of amino acids with sugars has received increasing attention, especially with respect to the non-enzymatic "browning" of foodstuffs. This reaction is of great interest to the food industry because of its relation to discoloration, palatability and the general deterioration that may occur during the processing and storage of food materials. However, the importance of this reaction with respect to its influence on the nutritive quality of food proteins has not been fully appreciated.

Recently Patton and his co-workers ('48a, b, c) have reported on the inactivation of free amino acids resulting from glucose-heat treatment, and have demonstrated that the amino acids in intact proteins are similarly attacked. McInroy et al.

[1] The material reported here is part of a thesis submitted by Leo Friedman in partial fulfillment of the requirements for the Ph.D degree in Biochemistry in the Graduate School of Georgetown University, Washington, D. C.

('49) have reported that the nutritive value of casein is seriously impaired after autoclaving with glucose.

In the course of our work on biological methods for evaluating the nutritive quality of protein hydrolysate preparations, it was observed that the presence of glucose resulted in marked deterioration of nutritive value under relatively mild conditions.

TABLE 1

Basal diet

CONSTITUENTS	AMOUNT	VITAMINS ADDED		
	gm		*per 100 gm diet*	*To prepare for 25 kg of diet*
Dextrin + vitamins	52	Thiamine HCl	200 μg	50 mg
Dextrin [1]	788-x	Riboflavin	300 μg	75 mg
Salts I U.S.P.	40	Niacin	1,500 μg	375 mg
Cod liver oil	20	Pyridoxine HCl	250 μg	63 mg
Wheat germ oil	10	Ca pantothenate	2,000 μg	500 mg
Cottonseed oil	90	Choline chloride	0.10 gm	25 gm
Test material (or casein) [2]	x	Dextrin	50 gm	1,250 gm
	1,000			

[1] Corn starch suspended in distilled water to a thin paste, autoclaved for two hours at 15 lb., broken into small pieces, dried at 50°C. and ground.

[2] To supply nitrogen at a level of 1.455%.

METHOD

The nutritive quality of protein hydrolysate preparations was evaluated by comparison with casein in a rat growth procedure similar to that recommended by Cannon and Cox (Nelson, '47). The protein-free basal diet is shown in table 1. The water-soluble vitamins were added in solution to a portion of the dextrin, dried at 40°C. and stored at 10°C. The test materials were added at the expense of an equivalent amount of dextrin, so that each 11 gm of diet furnished 0.16 gm of nitrogen, i.e., the diet contained 1.45% N. The casein used as a reference standard was prepared from fresh skimmed milk by isoelectric precipitation with dilute HCl and three reprecipitations from dilute ammonia solution, with washings after each precipitation.

Young white rats from our stock colony, weaned at weights ranging from 40 to 60 gm, were divided into groups of at least 8 animals per group. The groups were strictly comparable as to age, weight, sex and litter. The animals were kept in individual cages, on raised screens. Water was supplied ad libitum. The test diets were fed from the first day. Each animal was offered only 11 gm of food each day. The food intake was recorded daily and the animals weighed at regular frequent intervals. The feeding was continued for 28 days in most cases. The diets were compounded at least every other week and stored at 10°C.

Each test included a group receiving the reference casein. At the end of the assay period protein efficiency ratios were calculated for each group and the value of each test material relative to casein, considering casein as 100, was calculated. A typical assay is shown in table 2.

EXPERIMENTAL AND RESULTS

Two samples of a commercial protein hydrolysate powder, designated PH III and PH IV, were used. These materials were dried pancreatic hydrolysates of casein (total N 12.7% and amino N 7.8%), used commercially both for oral feeding and for the preparation of solutions for parenteral administration. The samples were taken into solution, with and without glucose, then dried in the following ways:

1. PH III Dried A: Fifty grams of the protein hydrolysate powder were dissolved in 1,000 ml of distilled water, immediately transferred to a vacuum still, and concentrated to a volume of approximately 250 ml at 50 to 60°C. during three hours. The concentrated solution was poured onto 150 gm of dextrin and dried in a forced draft oven at 45 to 60°C. for 36 hours. The dried preparation was ground and nitrogen was determined on an air dry sample (2.64% N). There was no change in color.

2. PH III + Glucose Dried A: Fifty grams of the protein hydrolysate powder and 50 gm of glucose were dissolved in 1,000 ml of distilled water and the solution concentrated and

TABLE 2

Assays for nutritive value

GROUP		I		II			III			IV			V	
Test protein		PH IV (powder) + glucose solutions dried in various ways								PH IV (powder)			Casein	
		A		B			C							
Initial wt. Av. (gm)		45.3		45.9			45.1			45.9			45.5	
		28-Day weight gain and food intake (gm)												
Litter	Sex	Gain	F.I.[1]	Sex	Gain	F.I.	Sex	Gain	F.I.	Sex	Gain	F.I.	Sex	Gain	F.I.
1	F	24	216	M	15	166	F	37	254	F	57	284	M	82	289
2	M	42	231	F	28	227	M	37	211	M	51	221	F	66	263
3	F	60	259	F	29	219	M	47	230	F	53	220	M	74	278
4	M	50	255	M	29	215	F	42	254	M	51	241	F	79	295
5	M	42	224	F	24	205	M	34	174	F	60	244	M	82	285
6	F	39	201	M	38	233	F	27	179	M	69	266	F	76	275
7	M	47	211	F	21	164	M	32	179	F	54	230	F	73	267
8	F	53	249	M	37	204	F	42	240	M	59	223	M	90	291
Av. gain/day		1.59			0.987			1.33			2.03			2.78	
Av. F.I./day			9.24			7.29			7.68			8.61			10.01
Av. food eff. gain/F.I.			0.193			0.135			0.173			0.235			0.277
Av. protein eff. gain/pro. I.			2.12			1.49			1.90			2.59			3.05
Nutritive value:															
By growth			57.4			35.5			47.9			73.0			100
By P.E.[2]			69.7			48.7			62.5			84.8			100
By P.E.[3]			82.1			57.4			73.6			100			...
% loss on drying			17.9			42.6			26.4			...			...

[1] Food intake.
[2] Referable to V as 100.
[3] Referable to IV as 100.

dried in the same manner as PH III Dried A. Nitrogen determined on an air dry sample was 2.64%. The color of the preparation was reddish brown.

3. PH III Dried B: A 5% solution of the protein hydrolysate powder was evaporated in a strong current of air to a thick syrup. The temperature of the solution during the 18-hour period of evaporation was 15°C. The syrup was mixed with dextrin and dried in a forced draft oven at 50°C. for 36 hours. The nitrogen content of the final preparation was 3.84% and there was no noticeable change in color.

4. PH III + Glucose Dried B: A 5% solution of the protein hydrolysate powder in 5% glucose was evaporated and dried in the same manner as PH III Dried B. Nitrogen determined on an air dry sample was 2.39%. The preparation was red brown in color.

5. PH III + Glucose Dried C: An attempt to dry a freshly prepared 5% hydrolysate in a 5% glucose solution from the frozen state was unsuccessful, and the partially dried material was mixed with dextrin and dried in the forced draft oven at 50°C. for 60 hours. The final preparation had a deep red brown color and contained 2.78% nitrogen.

6. PH IV + Glucose Dried A: A freshly prepared solution (5% PH IV powder plus 5% glucose) was evaporated to a syrup in the same manner as number 3 above. The syrup was mixed with dextrin and partially dried in a vacuum oven at 50°C. and finally in a vacuum desiccator at room temperature. The preparation was slightly brown and contained 2.56% nitrogen.

7. PH IV + Glucose Dried B: Prepared in the same manner as number 6 but accidentally overheated at one stage. The preparation was brown and contained 2.56% nitrogen.

8. PH IV + Glucose Dried C: A freshly prepared solution (5% PH IV + 5% glucose) was poured on dextrin and evaporated in a strong current of air, as in number 3. The final drying was in a vacuum desiccator at room temperature during a period of 8 days. The product was light brown in color and contained 3.03% nitrogen.

[1] Referable to IV as 100.

These 8 preparations and the original protein hydrolysate powders were assayed for their nutritive value by the procedure described above. The results are presented in table 3. The drying procedures produce a small loss, of questionable significance, in the nutritive value of a protein hydrolysate solution. In the presence of glucose there is a marked deterioration of biological value, ranging from 18 to 100%, depending on the conditions of drying, particularly temperature and duration of heating.

TABLE 3

Effect of drying protein hydrolysate solutions, with and without glucose, upon their nutritive value

SAMPLE	NUTRITIVE VALUE	LOSS ON DRYING
		%
Casein	100	...
PH III (powder)	93.6 90.9 av. 92.3	
PH III (powder) Dried A	88.7	3.9
PH III (powder) Dried B	85.8	7.0
PH III (powder) + Glucose Dried A	59.6	35.4
PH III (powder) + Glucose Dried B	0.0	100
PH III (powder) + Glucose Dried C	26.7	71
PH IV (powder)	84.8	...
PH IV (powder) + Glucose Dried A	69.7	17.8
PH IV (powder) + Glucose Dried B	48.7	42.6
PH IV (powder) + Glucose Dried C	65.2	26.3

The preparation PH III + Glucose Dried C did not support growth in young rats during the first week when fed at a level supplying 1.45% nitrogen in the diet. During the following 10 days the average growth of 6 rats on this diet was 6 gm. During the same period two rats of the same group received this preparation at a level of 2.12% N and the average growth was 6 gm. Two other rats from this group received, in addition to the original diet, 5% of a lactalbumin preparation (total N of this diet was 2.05%); the average growth on this supplemented diet was 13 gm. These results indicated that the loss of nutritive value during drying in the presence of glucose was prob-

ably not due to the formation of a toxic substance during the heating process, but rather to a deficiency which could be corrected by supplementation with good quality protein.

In order to determine whether or not the amino acids of the protein hydrolysate had been damaged by drying in the presence of glucose, experiments involving supplementation with purified amino acids were carried out. The addition of tryptophan and methionine individually did not result in an improved growth response. It became apparent that if, as a result of the drying process, a deficiency of more than one amino acid had developed, supplementation with individual amino acids would not make up the deficit. Supplementation with mixtures of amino acids would involve an impractically large number of combinations. However, if a mixture of the 9 amino acids essential for the growth of the rat were added to the deficient diet, a good growth response would be expected. Then, by omitting one amino acid at a time from this combination, it would be possible to identify the amino acids missing from the inadequate diet. Accordingly, 10 diets were prepared, each containing PH III + Glucose Dried C at a level of 1.45% N. To each 10 gm of one of the 10 diets were added 17 mg L-histidine HCl, 50 mg DL-isoleucine, 90 mg L-leucine, 50 mg L-lysine HCl, 25 mg DL-methionine, 40 mg DL-phenylalanine, 30 mg DL-threonine, 15 mg L-tryptophan, and 50 mg DL-valine. One of these amino acids was omitted from each of the other diets. Two weanling rats were maintained on each of these diets during a period of 5 days. The growth curves for this period are given in figure 1. Although the experiment was of short duration and only two animals were used in each group, it is clear that the amino acids histidine, threonine, phenylalanine, tryptophan and lysine are adversely affected by the treatment with glucose, and this is true to a lesser extent of the other amino acids as well.

This experiment was repeated with a preparation obtained by drying a 5% hydrolysate (PH IV), 5% glucose solution. Groups of 5 weanling rats were fed each diet for a period of 12 days. All the animals were maintained for two days on the

basal diet before the feeding of the supplements was started. The results are presented in table 4. With the possible exception of methionine, the previous findings were corroborated. It is clear that threonine, phenylalanine, methionine, lysine

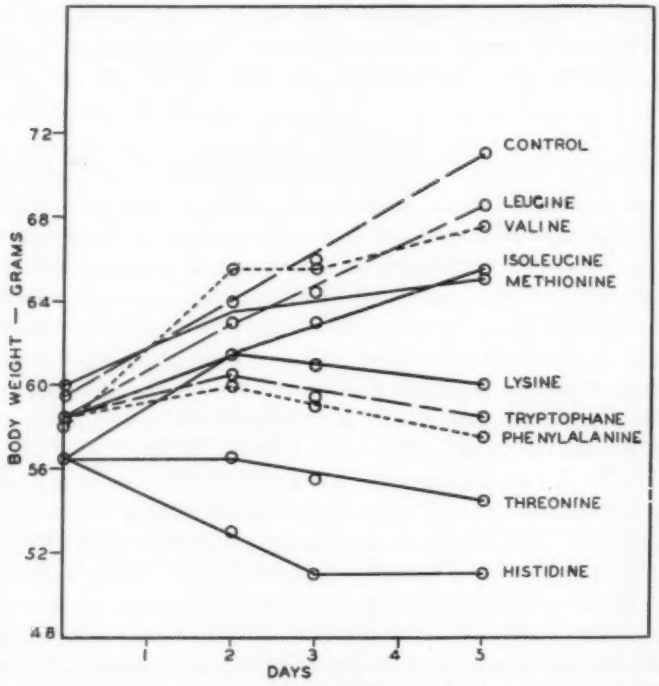

Fig. 1 Growth response to amino acid supplementation of a glucose-treated protein hydrolysate. Control fed 9 essential amino acids. In the other cases the amino acid indicated is omitted. Two rats in each group.

and histidine are involved in the reaction with glucose. Unquestionably, tryptophan also is affected. It may be that hydrolysate PH IV has a higher tryptophan content than PH III. The results of these feeding experiments do not preclude the possibility that isoleucine, leucine and valine also are involved.

The original protein hydrolysate powder (PH III) and sample 5 (PH III + Glucose Dried C) were assayed for the 10 essential amino acids by the microbiological method of Stokes et al. ('45). The samples were dissolved in water and assayed without any further treatment. The results, expressed as milligrams of amino acid per gram of nitrogen, are shown in table 5. The per cent change in each amino acid from the value in the original powder (PH III) is also given, and the amino

TABLE 4

Effect of supplementing glucose-treated protein hydrolysate with amino acid mixtures. Average change in weight and food intake during 12-day feeding period. (Each value is the average of 5 rats)

DIET	CHANGE IN WEIGHT	FOOD INTAKE
	gm	*gm*
Basal diet + 9 amino acids	10.0	67.4
Isoleucine [1]	4.6	44.8
Leucine [1]	11.8	60.6
Tryptophan [1]	6.0	58.0
Valine [1]	5.4	54.4
Histidine [1]	— 2.6	28.2
Lysine [1]	— 2.8	49.0
Methionine [1]	— 4.0	50.8
Phenylalanine [1]	— 2.4	41.8
Threonine [1]	— 3.2	44.6
Basal diet	— 5.2	48.6
Basal diet + PH IV powder (8%)	11.8	72.6

[1] Basal diet (table 1), including PH IV (powder) dried with glucose supplemented with mixtures of amino acids as indicated in text; in each case the amino acid listed being omitted.

acids are tabulated in order of increasing loss in sample 5 (PH III + Glucose Dried C), which has no nutritive value for growth. It is interesting that the order of the amino acids corresponds closely to the order obtained in the animal experiments as shown in figure 1. Both the biological and microbiological experiments are in agreement that the greatest changes occurred in the amino acids histidine, threonine, lysine, tryptophan and phenylalanine, and that the other amino acids were also affected but to a lesser degree.

In table 6 are shown the quantities of essential amino acids, as calculated from the microbiological assay data, that are supplied each rat daily by the protein hydrolysate before and after drying in the presence of glucose.

TABLE 5

Amino acid composition[1] *of a protein hydrolysate powder, before and after drying in the presence of glucose*

AMINO ACID	PH III (POWDER)	PH III (POWDER) + GLUCOSE DRIED B	
	mg/gm N	*mg/gm N*	*% loss*
Isoleucine	417	397	5
Leucine	634	591	7
Valine	442	402	9
Arginine	246	217	12
Methionine	208	163	18
Phenylalanine	360	296	22
Tryptophan	67	52	22
Lysine	720	560	22
Histidine	240	170	29
Threonine	298	212	29
Tyrosine	75	49	35
Nutritive value rel. to casein	91	0	

[1] Method of Stokes et al. ('45).

TABLE 6

Intake of amino acids per rat when samples were fed at the level of 0.16 gm N per day

AMINO ACIDS	PH III (POWDER)	PH III (POWDER) + GLUCOSE DRIED B
	mg	*mg*
Histidine	38.4	27.2
Isoleucine	66.7	63.5
Leucine	101.4	94.6
Lysine	115	90
Methionine	33	26
Phenylalanine	58	47
Threonine	48	34
Tryptophan	11	8
Valine	71	64
Arginine	39.4	34.7
Nutritive value rel. to casein	91	0

A most interesting aspect of the data in tables 5 and 6 was the small differences in amino acid composition that resulted in marked differences in nutritive value, ranging from excellent quality to complete nutritional failure.

DISCUSSION

The work reported here affords direct evidence that the amino acid-sugar reaction has a profound effect on the nutritive value of protein hydrolysates for the rat, and that most of the dietary essential amino acids are made unavailable by the reaction. Hsu et al. ('48) have recently reported that autoclaving a soybean protein with glucose damaged the protein to the extent that supplementary methionine, lysine and tryptophan failed to restore its nutritive value for chicks, indicating that other amino acids in addition to these three were destroyed or rendered unavailable. Furthermore, acid hydrolysis of an autoclaved mixture of glucose and methionine failed to alter the degree of availability of the amino acid for the support of chick growth.

It is of special interest to note that relatively small changes in amino acid composition, as measured by microbiological assay, are associated with important changes in nutritive value for the rat. The daily intake of essential amino acids from a diet containing the glucose-treated protein hydrolysate that did not support growth was calculated from microbiological assay data, as is shown in table 6. The amounts of essential amino acids consumed were within the range of the daily requirement of the rat and would be expected to permit growth. This is borne out by a preliminary experiment in which pure amino acids, fed in a mixture equivalent to the essential amino acid composition of the inadequate diet, promoted an appreciable growth response. This would exclude the possibility that the discrepancy noted is the result of non-availability of the dispensable amino acids, e.g., glutamic acid, present in the hydrolysate. It appears, therefore, that there is an important difference between the animal and the microorganism with

respect to the nutritional availability of the amino acids after their reaction with glucose. This raises the question of the applicability of the results of the microbiological assay of amino acids in assessing the nutritive quality of protein materials that have been subjected to heat treatment. Mitchell and Block ('46) have presented instances, especially in the case of cereal proteins, where the nutritive value was impaired by heat with no demonstrated alteration in the content of the essential amino acids as measured by chemical methods.

It is suggested that the formation of an amino acid-glucose complex that is not readily utilized by the animal may be the basis for the difference noted in the experiments reported here.

SUMMARY

In the presence of glucose the nutritive quality of protein hydrolysate solutions deteriorates. Extensive decreases in biological value upon drying such solutions are accompanied by "browning" of the preparations. The intensity of the color is to some degree related to the extent of deterioration of the nutritive quality.

The reduced biological value results from the reaction of amino acids with glucose. Feeding experiments with pure amino acid supplements indicate that histidine, threonine, tryptophan, phenylalanine, lysine and methionine become unavailable to the animal. Leucine, isoleucine and valine were not appreciably affected.

Relatively small losses in amino acids as measured by microbiological assay were associated with drastic changes in nutritive value for animals. There appears to be an important difference in the nutritional availability to animals and to microorganisms of the amino acid-glucose reaction products. It is suggested that an amino acid-glucose complex is formed which is unavailable to the rat but relatively more available to the microorganism. This raises the question of the validity with respect to nutritional problems of amino acid microbiological assay values for heat damaged proteins.

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PYRIDOXINE DEFICIENCY IN THE CALF

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THREE FIGURES

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A "synthetic milk" ration has been developed which is satisfactory for the normal growth of day-old calves (Wiese et al., '47a). Using this diet, it has been found that the bovine requires, either preformed in its diet or from rumen intestinal synthesis, riboflavin (Wiese et al., '47b), biotin (Wiese et al., '46) and thiamine (Johnson et al., '48a). A preliminary report on pyridoxine and pantothenic acid has appeared (Johnson et al., '47b). In addition, it has been found that on this "synthetic milk" ration, which contains 30% casein (dry basis), nicotinic acid is not required by the young calf (Johnson et al., '47a). In this paper we wish to report the deficiency symptoms appearing when pyridoxine is omitted from the ration.

EXPERIMENTAL AND RESULTS

The composition of the pyridoxine-deficient "synthetic milk" ration is given in table 1. The experimental animals, 8 male calves, were housed and fed as previously reported (Wiese et al., '47a). Three calves (two Holsteins and one Guernsey) served as positive controls and received the ration given in table 1 plus 0.65 mg of pyridoxine per liter of milk. Four calves (one Holstein and three Guernseys) received the basal ration without supplement, and one calf (a Holstein) received the basal ration plus the anti-vitamin, desoxypyridoxine. Normal growth as compared to the Ragsdale standards (Ragsdale, '44) was obtained in the calves receiving pyri-

doxine, as is shown by the growth curves given in figure 1. The growth curves of the calves fed the pyridoxine-deficient diet are given in figure 2; the growth rates here are slower and more irregular, and in all cases there is a plateau in weight. The first two calves on the deficient diet were calves 43G and 42H. Calf 43G reached a plateau in weight after three and one-half weeks on the basal diet. His appetite was poor on the 23rd day, and he began to scour on the 27th day. On the 40th day he was still scouring, his appetite was very poor,

TABLE 1

Composition of "synthetic milk" ration

COMPONENTS	AMOUNT	VITAMINS IN MILLIGRAMS PER LITER OF "MILK," ADDED AT TIME OF FEEDING	
	%		
Casein (Labco)	30.0	Thiamine	0.65
Lard	31.0	Riboflavin	1.30
Mineral mixture [1]	6.0	Calcium pantothenate	5.2
Cerelose	33.0	Inositol	26.0
		Choline	260.0
		p-Aminobenzoic acid	2.6
		Pteroylglutamic acid	0.052
		Biotin	0.01
Vitamin A	5,000 I.U. per day	α-Tocopherol acetate [2]	1.0
Vitamin D	500 I.U. per day	2-Methyl-1,4-naphthoquinone [2]	0.26

[1] For the composition of the mineral mixture see Johnson et al., '48b.

[2] The vitamin E and vitamin K dissolved in a small amount of lard were homogenized along with the rest of the lard into the solution of casein, salts and cerelose.

and he was sluggish and lay down most of the time. On the 90th day this calf had an epileptiform fit (jumped in the air, tried to jump out of the cage, ran around in the cage, appeared "wild-eyed," and so forth). On the 96th day therapy was begun, with 100 mg of pyridoxine given per day. However, 4 days later the calf died during a second fit. Pathological examination showed hemorrhages of the epicardium and kidneys, mild to moderate demyelination of peripheral nerves, and moderate proliferation of Schwann cells, but no brain lesions.

Holstein calf 42H appeared more resistant to this deficiency but had almost reached a plateau in weight by 10 weeks. This calf began to scour at 22 days, and his appetite was very poor after 66 days on the pyridoxine-low basal ration. At 96 days his activity was decreased and his haircoat was poor and falling out. On the 104th day of age therapy was started. The calf was given orally 100 mg of pyridoxine per day, and three

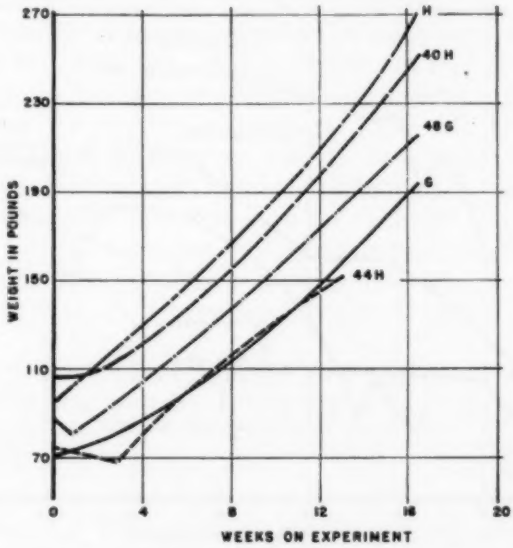

Fig. 1 Growth curves of calves receiving the "synthetic milk" ration supplemented with pyridoxine compared to Ragsdale "normals." Curves 40H (Holstein), 44H (Holstein) and 48G (Guernsey) are for the experimental calves. Curves H (Holstein) and G (Guernseys) are the Ragsdale normals.

days later he showed signs of improvement. At 4 days he showed increased activity, and after 14 days his haircoat appeared improved. However, the calf failed to gain and was taken off experiment after 4 weeks on therapy. Pathological examination of the lungs showed some degree of atelectasis and evidence of pneumonia, which may explain the lack of a more positive response to pyridoxine therapy. The sciatic

nerve showed moderate proliferation of Schwann cells and moderate to mild demyelination of myelinated fibers.

Guernsey calf 45G on the pyridoxine-free ration developed a poor appetite by the 28th day. His appetite became progressively worse, and at 7 weeks his weight had reached a plateau. At 73 days the calf appeared very sluggish, had started to scour and had developed a cough. Therapy was started at 80

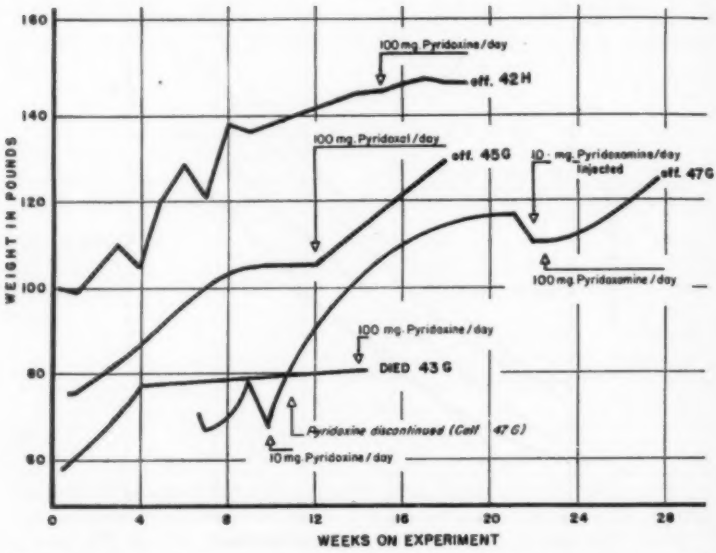

Fig. 2 Growth curves of calves receiving the pyridoxine-free "synthetic milk" ration.

days, and the calf was given orally 100 mg of pyridoxal [1] per day. In two days he was hungrier and friskier, and after 6 weeks, when taken off experiment, was apparently normal and doing well.

Guernsey calf 47G, also on the pyridoxine-free ration, developed anorexia in approximately two weeks, and at three weeks

[1] Pyridoxamine dihydrochloride, pyridoxal hydrochloride and desoxypyridoxine hydrochloride were very generously donated by Merck and Company, Inc., Rahway, New Jersey, through the courtesy of Dr. D. F. Green.

had lost weight due to his poor appetite. At this time (26 days) 10 mg of pyridoxamine [2] per day were given orally for 8 days. The calf showed an immediate improvement in appetite and appeared normal again. However, within two weeks after discontinuing therapy (50 days) his appetite was again poor, and at 58 days there was some scouring. At 82 days of age this calf had an epileptiform fit (as did calf 43G). It lay on its side with its head drawn forward onto its chest and with its feet kicking wildly. Pyridoxamine therapy was begun again on the 119th day. The pyridoxamine was given at the rate of 10 mg per day by subcutaneous injection for 5 days and 100 mg per day orally thereafter. The calf did show an increased appetite, scouring ceased, and there was some weight gain. However, he had apparently contracted an infection during his deficient period, as had calf 42H, and on pathological examination he was found to have severe catarrhal enteritis (a thick catarrhal exudate throughout the small intestine), which had prevented him from making a better recovery.

Figure 3 shows the weight data for Holstein calf 46H. This calf received the pyridoxine-free ration to which was added desoxypyridoxine,[3] an antimetabolite of pyridoxine (Ott, '46), at the rate of 1.3 mg per liter of "milk." This amount of desoxypyridoxine was doubled at 71 days and tripled at 97 days. From the 115th to 121st day the calf was given 10 mg of desoxypyridoxine per day intravenously instead of the oral dose. From the 121st to the 157th day he received 18 mg of desoxypyridoxine per day intravenously. During all this time this calf showed only the same symptoms as were shown by the other deficient calves, and the time of onset of the symptoms was approximately the same, e.g., poor appetite appeared on the 34th day, inactivity and sluggishness along with "grinding jaws" on the 40th day. These symptoms persisted, the appetite became worse, and there was considerable weight loss. On the 108th day the calf was transferred to a nipple

[2] See footnote 1, page 312.
[3] See footnote 1, page 312.

pail and began to eat a great deal better, probably because less exertion was required. With this method of feeding the calf regained his earlier weight loss, and then the weight reached a plateau again. At 144 days the calf swayed and fell in attempting to walk and showed poor muscular coordination. At 157 days the desoxypyridoxine was discontinued and the calf was given 100 mg per day of pyridoxamine orally. This

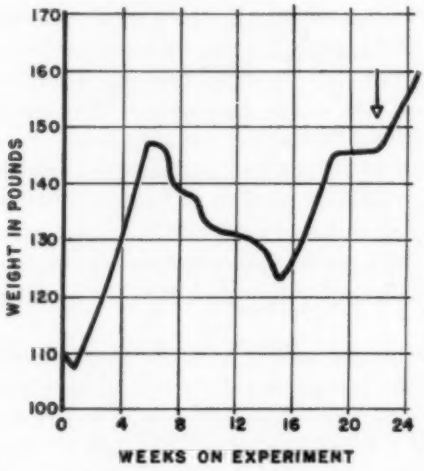

Fig. 3 Growth curve of calf 46H which received the pyridoxine-free "synthetic milk" ration plus desoxypyridoxine.

therapy resulted in an immediate increase in appetite and considerable weight gain over the next three weeks. However, the prolonged deficiency had apparently produced internal lesions, because on the 6th day of therapy the calf went into a convulsion or epileptiform fit with vigorous head retraction and violent thrashing of the legs while lying on his side. His breathing was rapid and forced. The attack lasted about 12 minutes, becoming less violent after 4 minutes. Ten minutes later the calf got up and was able to walk, although his muscles were twitching. Thirteen days later the calf had two

"fits" 20 minutes apart, each lasting 8 to 10 minutes. The next day the calf died during an attack.

It appeared that the increased food consumption and weight gains brought about by pyridoxamine therapy had brought out the deficiency symptoms not previously apparent due to the sluggish nature of the calf.

Pathological examination of this calf showed the following: *Heart.* Rather extensive hemorrhage in the sub-epicardial fat. Much of the myocardium showed evidence of retrograde changes, in that many of the fibers appeared to have lost their structure and the nuclei appeared pycnotic or showed karyorrhexis. *Radial nerve.* Evidence of mild proliferation of Schwann cells and considerable degeneration of nerve fibers was seen. *Small intestine.* Marked superficial desquamation was diffuse. *Large intestine.* Extensive desquamation of the epithelium occurred throughout the superficial half of the mucosa.

Twenty-four-hour urinary collections were made at approximately weekly intervals on all calves. Average representative excretion data for pyridoxine, pyridoxamine, and 4-pyridoxic acid are given in table 2. The total pyridoxine excretion (column 2, table 2), made up of pyridoxine, pyridoxal and pyridoxamine, was determined by the *Sacch. carlsbergensis* method of Atkin, Schultz, Williams and Frey ('43). The pyridoxamine excretion (column 4, table 2), made up of pyridoxamine and pyridoxal, was determined as pyridoxamine by the *Streptococcus faecalis* (American Type Culture Collection No. 8043) method of Rabinowitz and Snell ('47); 4-pyridoxic acid was determined by the method of Huff and Perlzweig ('44).

The excretion of all metabolites decreased on the pyridoxine-deficient diet as compared to the control ration. In particular, the pyridoxine excretion fell to approximately zero, the compounds determined by *Sacch. carlsbergensis* apparently being primarily pyridoxamine or pyridoxal, or both. The 4-pyridoxic acid excretion, on the other hand, remained around 1 mg per day, which, while lower than that of the controls, is still considerable. It is quite possible that in the case

of calf urine the assay procedure is not specific for 4-pyridoxic acid but is also measuring some extraneous material. However, the very marked rise in 4-pyridoxic acid excretion following pyridoxine, pyridoxamine or pyridoxal dosage shows that in the calf as in man (Huff and Perlzweig, '44; Johnson et al., '45) and in the rat (Sarma, Snell and Elvehjem, '46)

TABLE 2

Excretion of pyridoxine and metabolites

AGE IN DAYS	PYRIDOXINE, ETC.	4-PYRIDOXIC ACID	PYRIDOXAMINE, ETC.
	mg/day	*mg/day*	*mg/day*
	Ave. of 2 calves on "complete" ration		
3	. . .	1.64	0.035
16	0.136	3.39	0.091
30	0.56	2.16	....
92	1.2	4.70	....
	Ave. of 4 calves on B_6-free ration		
3	1.13	0.92	0.064
9	1.129	1.16	0.056
15	0.031	1.13	0.031
66	0.036	0.71	0.047
	After therapy (3 calves)		
	Pyridoxine, 100 mg per day; started on 104th day [1]		
122	19.36	60.4	5.95
	Pyridoxamine, 100 mg per day; started on 111th day [2]		
144	....	45.7	...
	Pyridoxal, 100 mg per day; started on 87th day [3]		
111	6.76	42.8	1.22

[1] Calf 42H. [2] Calf 47G. [3] Calf 45G.

4-pyridoxic acid is the chief metabolite of vitamin B_6. In these calves it accounted for 40 to 60% of the vitamin B_6 intake.

A disturbance in tryptophan metabolism resulting in the excretion of large amounts of xanthurenic acid occurs in pyridoxine-deficient rats (Lepkovsky and Nielsen, '42) and swine (Cartwright et al., '44), but not in dogs (Fouts and Lepkovsky, '42) and chicks (Lepkovsky et al., '43). Examination

and analysis for xanthurenic acid of the urines of both the pyridoxine-deficient calves and the control calves were made following tryptophan dosage. In no case was xanthurenic acid found to be excreted.

In view of the occurrence of microcytic anemia in pyridoxine deficiency in dogs (Fouts et al., '38) and the report of poikilocytosis in calves which responded to vitamin B_6 (Reid et al., '45), the blood picture of our calves was examined at frequent intervals throughout the experiment. A summary of the blood data is presented in table 3. No differences in blood picture appeared during pyridoxine deficiency. The calves showed no microcytosis, red blood cell diameter being approximately 5 μ in all cases. This is normal for calves (Kushner, '38). There was no evidence of poikilocytosis as reported by Reid et al. ('45) in any of our pyridoxine-deficient calves.

DISCUSSION

By using a "synthetic milk" ration devoid of pyridoxine a deficiency was produced in young dairy calves which responded to vitamin B_6 as pyridoxine, pyridoxal or pyridoxamine. Too few calves were used in testing each of the above for the differences in response to be significant. However, the most striking responses were on pyridoxal and pyridoxamine therapy. The deficiency symptoms produced in all cases included lack of appetite, anorexia, poor gain in weight, listlessness and general lack of condition in the animals. The most striking symptom, observed in only a few calves (three), was the appearance of epileptiform fits due to a nerve pathology. This symptom is interesting in that it has previously been reported for the dog (Fouts et al., '38; McKibbin et al., '39), the rat (Chick et al., '39, '40; Oleson et al., '39; Patton et al., '44; Lepkovsky et al., '42), the pig (Chick et al., '38; Wintrobe, '39; Wiese et al., '48), the chick (Jukes, '39), the duck (Hegsted and Rao, '45) and the turkey (Bird et al., '43).

It appears also that the metabolism of pyridoxine is similar in different species, in contrast to nicotinic acid metabolism,

TABLE 3

Blood data for calves[1]

CALF NO.	TREATMENT	HEMOGLOBIN	RED BLOOD CELLS	WHITE BLOOD CELLS	NEUTROPHILS	LYMPHOCYTES	MONOCYTES	EOSINOPHILS	BASOPHILS
		gm/ml							
42 H	Before pyridoxine	11.3 (10)	8,360,000 (2)	8,000 (2)	14 (1)	81	4	0	1
	After pyridoxine	10.6 (4)	....	...	..	..	..	..	..
43 G	Before pyridoxine	12.1 (9)	4,570,000 (1)	4,000 (1)	7 (1)	88	5	0	0
	After pyridoxine	10.7 (1)	....	...	..	..	..	..	..
45 G	Before pyridoxal	9.1 (11)	7,435,000 (4)	6,300 (4)	..	..	..	..	..
	After pyridoxal	8.8 (7)	8,920,000 (4)	11,200 (4)	22 (3)	66	9	3	0
47 G	Before pyridoxamine	12.2 (17)	10,270,000 (8)	9,900 (8)	23.5 (2)	68	7	1.5	0
	After pyridoxamine	10.1 (5)	11,030,000 (5)	11,200 (4)	25.5 (4)	72.5	2	0	0.2
46 H	Desoxypyridoxine	10.3 (21)	10,130,000 (12)	10,560 (12)	37 (7)	57	6	0.5	0
	Pyridoxamine	8.0 (3)	7,060,000 (3)	12,920 (3)	40 (3)	58.3	1.6	0.3	0
Controls									
48 G		12.1 (15)	9,080,000 (8)	7,560 (8)	25 (3)	70	3.3	7	0
44 H		10.7 (13)	9,470,000 (4)	10,800 (4)	..	..	..	..	..

[1] Figures in parentheses indicate number of determinations.

which varies widely with species (Johnson et al., '47a; Perlzweig et al., '49).

We are at present unable to explain the discrepancy between our inability to find any effect of pyridoxine deficiency on the shape or size of the red blood cells and Reid, Huffman and Duncan's report ('45) of poikilocytosis in dairy cattle cured by pyridoxine. The difference may be due to the difference in age of the animals, since we were using newborn calves.

SUMMARY

1. The bovine species requires vitamin B_6, supplied either in the diet or by rumen or intestinal symbiosis. On a pyridoxine-deficient diet calves do not grow, and the excretion of pyridoxine and its metabolites is lowered.

2. Pyridoxine deficiency in the young calf reared on a pyridoxine-low "synthetic milk" ration is characterized by lack of apppetite, anorexia, poor growth, sluggishness and listlessness, poor haircoat (dull and falling out), and in some cases by the appearance of severe epileptiform fits and death.

3. If administered early enough, the calves respond to vitamin B_6 given as pyridoxal, pyridoxamine or pyridoxine.

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[1] Figures in parentheses indicate number of determinations.

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PLASMA TOCOPHEROL IN DIABETES MELLITUS[1]

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The purpose of this communication is to report an analysis of values of plasma tocopherol in 63 cases of diabetes mellitus. The data were obtained in the course of a trial of vitamin E therapy in diabetes (Bensley et al., '49).

METHODS

Selection of cases

All cases were adult diabetics attending the Outpatient Department of the Montreal General Hospital. The mean, maximum and minimum ages were 59, 75, and 31 years respectively. There were 49 women and 14 men. Of the 63 cases, 55 made up the "vitamin E and placebo groups" used in our trial of vitamin E therapy (Bensley et al., '49). The remaining 8 cases were also selected for this trial but failed to follow the necessary instructions. In all cases, diabetes was under poor control, as was shown by hyperglycaemia, and frequently hypercholesterolaemia, in the fasting state. No subjects received supplements of tocopherol concentrates before or during the collection of the data reported here.

Collection and analysis of samples of blood

All samples of blood were collected in the morning in the fasting state. Estimations were made of plasma tocopherol

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(Quaife and Harris, '44; Quaife and Biehler, '45), plasma cholesterol (Bloor, '16; Rabinowitch, '29) and blood sugar (Myers and Bailey, '16).

Studies of food intakes

Dietary histories were taken in all cases. No subject was following the prescribed diet but there was little variation in food intakes from month to month.

TABLE 1

Results of repeated estimations of plasma tocopherol over periods of 5 to 8 months in diabetics not receiving tocopherol supplements

CASE NUMBER	NUMBER OF TESTS	PLASMA TOCOPHEROL	
		Maximum	Minimum
		mg%	mg%
11414/47	8	0.8	0.6
17925/41	10	0.9	0.8
4510/45	9	1.1	0.9
16482/42	8	1.7	1.2
16876/41	8	2.2	1.3
11339/45	10	2.2	1.9

RESULTS

Two hundred and seventy estimations of plasma tocopherol were made in 63 cases. The maximum, minimum and mean values and the standard deviation of the sample were 2.4, 0.5, 1.1 and $\pm$ 0.4 mg%, respectively. Except for the high maximum, these values are similar to those found in normal subjects (Lemley et al., '49).[2] The maximum value obtained by the technique of Quaife and Harris ('44) in normal subjects was 2.0 mg% (Harris et al., '46); values exceeding this occurred in 7 of our 270 tests on diabetics. Although the plasma tocopherol levels of the entire group

[2] We made 41 estimations of plasma tocopherol in 33 normal subjects. Maximum, minimum and mean values and the standard deviation of the sample were 1.6, 0.6, 1.0 and $\pm$ 0.2 mg% respectively.

ranged between 0.5 and 2.4 mg%, values in any one case usually showed little variation (table 1).

An attempt was made to calculate intakes of vitamin E on the basis of the dietary histories. It was found that presently available information concerning the tocopherol content of prepared foods is too limited to permit accurate calculations of dietary intakes of this vitamin. However, intakes

TABLE 2

Relation between plasma tocopherol, plasma cholesterol and blood sugar in diabetes mellitus

(Based on analyses of three blood samples in each of 48 cases)

PLASMA TOCOPHEROL	NUMBER OF TESTS	PLASMA CHOLESTEROL			BLOOD SUGAR		
		Mean	SD [1] sample	R [2]	Mean	SD [1] sample	R [2]
mg%		*mg%*	*mg%*		*mg%*	*mg%*	
0.6 — 0.8	38	195	± 33		215	± 54	
				7.4			0.7
0.9 — 1.2	58	231	± 37		210	± 53	
				9.3			2.9
1.3 — 2.4	48	291	± 57		236	± 78	

[1] SD = standard deviation.

[2] R= ratio of difference between means to probable error of difference.

were studied from the standpoint of their content of foods rich in vitamin E. No relation between food intakes and plasma tocopherol levels could be discovered. Similar findings were reported by Williams ('48).

Failure to discover a relation between plasma tocopherol levels and food intakes led us to search for some other factor which might account for the wide variations in plasma tocopherol among these subjects. Relations between plasma tocopherol, blood sugar and plasma cholesterol were explored. The results (table 2) show that levels of tocopherol and cholesterol in plasma are closely related,[3] but no significant relation was demonstrated between plasma tocopherol and blood sugar.

[3] This relationship is not due to interference by cholesterol with the estimation of tocopherol (Baxter, '47; Darby et al., '49).

DISCUSSION

Previously reported studies (Bensley et al., '49) have shown that plasma tocopherol levels in patients with diabetes mellitus can be raised by increasing tocopherol intake through oral administration of tocopherol concentrates. These elevations were not associated with significant changes in plasma cholesterol levels. It therefore appears that the tocopherol content of plasma, in diabetes mellitus, is related to at least two independent factors — tocopherol intake and level of plasma cholesterol. The influence of this latter factor may explain the failure to demonstrate a relation between plasma tocopherol and dietary intake of vitamin E in diabetics not receiving supplements of tocopherol concentrate. A similar relation between levels of tocopherol and cholesterol in plasma was reported by Darby et al. ('49) to obtain in a variety of conditions, which included diabetes with nephrosclerosis, carotinaemia with unexplained hypercholesterolaemia, xanthomatosis, cerebral thrombosis, coronary occlusion and carcinoma of the pancreas. It is obvious that this relation must be explored further before plasma tocopherol levels can be used to assess nutritional status with respect to vitamin E.

SUMMARY

An analysis of values of plasma tocopherol in 63 cases of diabetes mellitus is reported. The mean value was normal but values exceeding the maximum normal level were met with in 7 of 270 tests. Although the plasma tocopherol levels of the entire group ranged between 0.5 and 2.4 mg%, values in any one case usually showed little variation. Plasma tocopherol levels could not be related to intakes of foods rich in vitamin E but were related to levels of plasma cholesterol.

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